STEINERNEMA SCAPTERISCI NGUYEN AND SMART, 1990: BACTERIAL ASSOCIATES, CULTURE, AND PATHOGENICITY

By

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Major Department: Entomology and Nematology

The bacteria Xenorhabdus spp., X. bovienii, Pseudomonas aureofaciens, Ochrobactrum anthropi, Xanthomonas maltophilia and an unknown genus characterized as a Gram-negative coccus related to Paracoccus denitrificans, were associated symbiotically with third-stage infective juveniles of the entomopathogenic nematode Steinernema scapterisci. First stage juveniles which hatched from sterilized eggs developed to adults and reproduced on cultures of each of the bacteria. The nematode also developed and reproduced on cultures of Xenorhabdus nematophilus, a symbiont of S. carpocapsae, and on cultures of Pseudomonas fluorescens and Escherichia coli. The life cycle (egg to egg) was completed in four days on all bacteria except E. coli, on which it took five days. Reproduction was excellent on P. aureofaciens (> 6,400-fold),

X. nematophilus (> 5,900-fold), and P. fluorescens (> 3,900-fold). All the infective juveniles examined retained bacteria (5 to 60 cells per infective juvenile) in the intestinal vesicle regardless of the origin of the bacteria.

All Xenorhabdus species, P. aureofaciens, and E. coli were pathogenic to a mole cricket, Scapteriscus borellii or to the house cricket, Acheta domesticus. No differences occurred in mortality of S. borellii whether infective juveniles of S. scapterisci were reared in vivo or on monoxenic cultures with O. anthropi, P. aureofaciens, X. nematophilus or an unknown coccus (genus undetermined).

Of three culture media tested, production of S. scapterisci on X. nematophilus was best in a semi-solid dog food medium, producing an average of 3.8×10^4 juveniles/ml medium (96.7% infective juveniles) from an initial inoculum of 200 nematodes/ml.

The LD_{50} of S. scapterisci to Scapteriscus spp. was 4,000 infective juveniles, whereas the LD_{50} to a field cricket, Gryllus rubens, and the lubber grasshopper, Romalea guttata, was 1,000 infective juveniles.

The ability of S. scapterisci to reproduce and retain cells of several bacteria and to be as pathogenic to insects while having various bacterial associations, may contribute to its chances to survive in nature and compensate for the low number of bacterial cells retained by infective juveniles.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Nematodes of the genus Steinernema Travassos, 1927 (Rhabditida:Steinernematidae) have been the subject of intense research throughout the world as attractive biological control agents of insect pests in agriculture. Such enormous interest is due to the many positive attributes of these entomophagous nematodes. Steinernematid nematodes, presently represented by nine described species (Poinar 1990), are either potential parasites as indicated by laboratory inoculations, or natural parasites of insects in ten insect orders (Poinar 1979). With such a wide host range, however, these nematodes seem not to pose more than a slight potential threat to populations of non-target insects (Kaya 1985).

Steinernema, like the closely related genus Heterorhabditis (Rhabditida:Heterorhabditidae), lives in association with bacteria, forming an association defined as symbiosis. The bacteria provide nutritional and reproductive factors for the nematodes (Akhurst 1983b, Poinar and Thomas 1966), which, in turn, transport the bacteria to the hemocoel of host insects where the hemolymph is a suitable substratum for bacterial multiplication (Poinar 1966). In addition, the nematodes produce a toxin or a factor which protects the bacteria against the host defenses (Poinar 1979, Gotz et al.

1981, Burman 1982). Third-stage nematode juveniles carry the bacterial cells in a vesicle located at the anterior portion of the intestine (Bovien 1937, Poinar 1966, Poinar and Leutenegger 1968, Akhurst 1982, Bird and Akhurst 1983). Within five hours after the nematodes penetrate into the insect hemocoel, the bacterial cells are released (Poinar 1966, Dunphy and Thurston 1990), multiply quickly and kill the host in 24 to 48 hours (Poinar 1979, Poinar and Hansen 1986). In this characteristicaly symbiotic association, the bacteria are not only a source of nutrients but also an inhibiting factor of other microorganisms (Poinar 1966; Poinar and Thomas 1966). Xenorhadus spp. are known to occur in two distinct phases, the primary and the secondary (Akhurst 1980), which show distinct characteristics concerning dye adsorption, pigmentation, production of antibiotics and lecitinase, presence of proteinaceous inclusions, and cell size (Boemare 1983; Boemare et al. 1983, Boemare and Akhurst 1988; Couche et al. 1987). Pathogenicity seems not to be affected but nematode reproduction in some nematode species associations is decreased in the presence of the secondary form of Xenorhabdus (Akhurst and Boemare 1990).

Associations between nematodes and bacteria have long been recognized. Bovien (1937) was the first to observe cells of bacteria in the anterior portion of the intestine of Steinernema (=Neoaplectana). Bovien visualized the importance of this association but did not try to characterize it. The

bacterium remained undescribed for many years until Poinar and Thomas (1965) proposed the name Achromobacter nematophilus. Since the genus Achromobacter was rejected (Hendrie et al. 1974), Thomas and Poinar (1979), proposed the genus Xenorhabdus in the family Enterobacteriaceae and transferred A. nematophilus to it. That genus initially comprised only two species, X. nematophilus associated with species of Steinernema and X. luminescens associated with species of Heterorhabditis Poinar, 1976.

Subsequent studies of bacteria associated with Steinernema and Heterorhabditis led to the recognition of four subspecies of Xenorhabdus nematophilus by Akhurst (1983a, 1986a, 1986b). Akhurst and Boemare (1988) later proposed that those subspecies be raised to the species level. Presently, described species of Xenorhabdus and the nematode species associated with them are as follows:

Xenorhabdus nematophilus, symbiont of Steinernema carpocapsae (Weiser, 1955) Wouts, Mracek, Gerdin, and Bedding, 1982.

Xenorhabdus bovienii, Akhurst & Boemare symbiont of S. feltiae (Filipjev, 1934), Wouts, Mracek, Gerdin and Bedding, 1982; of S. affinis (Bovien, 1937) Wouts 1991; of S. intermedia (Poinar, 1985) Wouts, 1991; and of some undescribed species of Steinernema.

Xenorhabdus poinarii, Akhurst & Boemare symbiont of S. glaseri Steiner, 1929 and an undescribed species of Steinernema.

Xenorhabdus beddingii, symbiont of two Steinernema spp.

Xenorhabdus luminescens, symbiont of Heterorhabditis spp.

The symbionts of S. anomali (Kozodoi, 1984) Wouts, 1991, and S. rara (Doucet, 1986) Wouts, 1991 are undetermined species of Xenorhabdus.

The relationship between these species of bacteria and Steinernema was first described by Poinar (1966). Bacterial cells were shown to be present in the anterior portion of the intestine of third stage juveniles of S. carpocapsae. The importance of bacteria for the development of steinernematid nematodes was elucidated by Poinar and Thomas (1966). This association known as mutualism (Poinar 1979) has been emphasized in many other papers (Akhurst 1982, 1983b, 1986a, 1986b, Boemare 1983, Boemare et al. 1983).

It has been suggested that Xenorhabdus does not have the ability to survive in nature (Poinar 1979). Except for one paper in which X. luminescens was reported from human clinical specimens (Farmer et al. 1989), Xenorhabdus has been reported only from infective juveniles of steinernematids and heterorhabditids or from insects infected by either nematode. Although Xenorhabdus is not able to infect insects when ingested or applied topically, it is highly pathogenic when

introduced into the insect hemolymph (Poinar and Thomas 1967, Milstead 1979).

Associations with bacteria other than Xenorabdus have been reported previously. Weiser (1963) isolated species of Pseudomonas from S. carpocapsae (DD-136) and concluded that those bacteria multiplied and caused the death of the insects (Weiser 1963). Poinar (1966) observed several species of bacteria that are commonly found in the gut of healthy Galleria mellonella (L.) larvae. Pseudomonas aeruginosa (Schr.) Migula, Proteus sp., Alcaligenes sp. and Aerobacter sp. may also develop along with infective juveniles of S. carpocapsae in the hemocoel of dead G. mellonella larvae. Poinar suggested that although the nematodes fed indiscriminately on all bacteria present, the infective juvenile could retain only Xenorhabdus nematophilus. He was able to detect P. aeruginosa and X. nematophilus in the hemolymph of nematode-infected hosts, and concluded that P. aeruginosa probably entered the host while attached to the nematode cuticle. Although Poinar reported exceptions, he considered pseudomonads non-pathogenic to insects. Poinar and Thomas (1966) also observed that other bacterial species may be suitable for reproduction of the nematode although reproduction improved when Xenorhabdus was the dominant microbe.

Lysenko and Weiser (1974) examined the microflora of Steinernema (=Neoaplectana) carpocapsae and observed that

Alcaligenes odorans (Malek and Kazdova-Koviskova 1946) Malek, Radochova and Lyzenko 1963, Pseudomonas maltophilia, P. alcaligenes Monias 1928, and Acinetobacter sp. were associated with the nematodes in large quantities. Pseudomonas sp., Alcaligenes sp., Achromobacter sp., and Flavobacterium sp., also were associated with S. carpocapsae but at a lower frequency. Pseudomonas maltophilia and Alcaligenes odorans were present on the nematode surface and in the gut, but the number of cells in the gut was much lower than the number on the nematode surface. Pseudomonas fluorescens (Trevisan 1889) Migula 1895 also was carried in the gut by some of the nematodes examined in this study. When testing pathogenicity of some bacteria, Lysenko and Weiser (1974) found that P. fluorescens presented an LD_{50} similar to that of Xenorhabdus (=Achromobacter) nematophilus. Axenic nematodes inoculated with Xanthomonas (=Pseudomonas) maltophilia caused the highest mortality to G. mellonella larvae.

Boemare et al. (1983) were able to demonstrate that Steinernema (=Neoaplectana) carpocapsae could grow and reproduce on cultures of Enterobacter aglomerans Beijerinck 1888) Ewing and Fife 1972, Serratia liquefasciens (Grimes and Hennerty 1931) Bascomb, Lapage, Willcox and Curtis 1971, and P. fluorescens. Infective juveniles obtained from such associations were shown to be pathogenic to G. mellonella and reproduced on that host. Those authors found that X. nematophilus acts as a nutrient source, as a factor for

nematode reproduction, and as a regulator of other species of bacteria.

Steinernema scapterisci Nguyen and Smart, 1990 was introduced into Florida in 1985 (Nguyen and Smart 1988, 1990a) as part of a project of the University of Florida for the biological control of mole crickets (Scapteriscus spp.). The nbiology of this nematode species was studied by Nguyen and Smart (1990a, 1990 b, 1991a, 1991b) but information concerning associations with bacteria are lacking. Knowledge of these associations is essential to culture the nematode and to better understand its parasitic action.

The objectives of this work were to determine the bacterial symbionts of S. scapterisci, to determine the development and reproduction of the nematode in monoxenic culture with various bacterial species, to determine the pathogenicity of these bacteria to selected insects, to determine the ability of infective juveniles to retain these bacteria and become infective and to improve methods to culture S. scapterisci in vitro.

CHAPTER 2
BACTERIAL SYMBIONTS OF *STEINERNEMA SCAPTERISCI*

Introduction

It has been demonstrated in Florida, (Smart et al. 1991) that *Steinernema scapterisci* is a good biological control agent of mole crickets (*Scapteriscus borellii* Giglio-Tos and *S. vicinus* Scudder). Soon after its introduction in 1985 from Uruguay, and some field applications, *S. scapterisci* became established and has been disseminated naturally to larger areas (Frank et al. 1990, Smart et al. 1990). Widespread dissemination is likely due to the active behavior of mole crickets, which can migrate even after they are infected with nematodes.

The symbiont of *S. scapterisci* remained unknown despite the characterization presented by Nguyen (1988) of a bacterium isolated from infective *S. scapterisci* juveniles. An attempt to classify the bacterium was not made, although some biochemical and cultural characteristics were presented. Those data do not completely match the description of *Xenorhabdus* spp.

Knowledge of the bacterial symbionts of *S. scapterisci* is necessary and desirable for producing the nematode on culture medium and to better understand its parasitic action on

insects. The objective of this work was to determine which bacteria are associated with S. scapterisci from various origins.

Materials and Methods

These studies consisted of the isolation and identification of bacteria living in association with Steinernema scapterisci obtained from various insects and environments. Nematodes isolates were obtained from the following sources:

1. From cultures continuously propagated in vivo on mole crickets (Scapteriscus borellii and S. vicinus) and house crickets (Acheta domesticus) at the Entomology and Nematology Department, University of Florida, Gainesville, FL.

2. From mole crickets (Scapteriscus spp.) captured at the Green Acres Agricultural Farm, University of Florida, Alachua County, FL, and subsequently cultured in the laboratory on mole crickets (S. borellii and S. vicinus) and on house crickets (A. domesticus).

3. From mole crickets (Scapteriscus sp.) captured near LaCrosse, FL, and subsequently cultured in the laboratory on mole crickets (Scapteriscus borellii and S. vicinus) and house crickets (A. domesticus).

4. From in vitro cultures at the Entomology and Nematology Department, University of Florida, Gainesville, FL.

Steinernema carpocapsae strain All was used as a source of the bacterium Xenorhabdus nematophilus, which was used as a reference strain. Infective juveniles of S. carpocapsae were obtained from cultures on Galleria mellonella. Infective juveniles of S. scapterisci and S. carpocapsae were stored in water suspensions at 8C for a maximum period of two months.

Isolation of bacteria

Infective juveniles were rinsed with sterile deionized water and transferred to a solution composed of 0.1% merthiolate and 0.6% streptomycin sulphate and maintained for 12 hours under aseptic conditions. The nematodes then were rinsed three times with sterile water and transferred, as a suspension in sterile deionized water, to a tissue grinder where they were crushed by friction. The resulting suspension, consisting of nematode tissue and bacterial cells, was pipetted to 9-cm plastic petri dishes containing either 8g Nutrient Agar (Difco Nutrient Broth, Difco Laboratories, Detroit, MI; 15 g agar, 1,000 ml deionized water) or T7 - TTC medium (5 g peptone, 3 g yeast extract, 10 g lactose, 0.1 ml tergitol, 0.025 g bromothymol blue, 0.04g triphenyltetrazolium chloride, 15 g agar, 1,000 ml deionized water). The petri dishes were maintained in an incubator at 25C and observed daily. After 48 hours, bacterial isolates were subcultured by transferring single colonies showing any morphological differences (size, shape, growth rate, or color) which might indicate a different species or genus.

Identification of bacteria

For comparisons of the bacterial isolates, reference cultures of Xenorhabdus nematophilus (ATCC 19061) and Xenorhabdus bovienii (ATCC 35271) were obtained from the American Type Culture Collection (Rockville, MD). Identification of all bacterial isolates was conducted at the Plant Pathology Department, University of Florida, Gainesville, FL, by Ms. Nancy C. Hodge. Pure cultures submitted for analysis were grown on trypticase soy broth agar (30 g trypticase soy broth, 15 g agar) and maintained at 29C for 24 $\pm$ 2 hours.

Preliminary identification was done by fatty acid (FA) analysis using the Microbial Identification System (MIDI, Newark, DE). Cellular fatty acid biosynthesis is a stable genetic trait which is highly conserved (Sasser 1990). The presence, absence and ratios of acids provide an array of data for distinguishing bacteria. FA profiles of unknown organisms are compared to those of reference strains stored in the MIDI library database. The MIDI pattern recognition programs determine the closest match, which is reported as similarity index (SI), ranging from 0.000 to 1.000. An SI of 0.6 to 1.0 is considered to be an excellent match.

Two graphic representations also can be used to determine multiple relationships between strains. Similarities are established in a dendrogram based on the Euclidian distance among selected strains which are simultaneously compared. An

Euclidian distance of 6.0 or less defines similar subspecies or biotypes, of 10 or less defines similar species, and of 25 or less similar genera. In addition, 2-D plots can be used for establishing similarities. In this type of graphic, the X axis represents principal component 1 which is responsible for 95% of the variation, and the Y axis represents principal component 2 which represents 5% of the variation. Comparisons then can be established depending on the location of the strains in the graphic.

Biochemical assays to confirm the MIDI identification were performed on many of the isolates. The tests included: Hugh-Leifson for aerobic/anaerobic respiration, Kings Medium B for fluorescence, oxidase, and arginine dihydrolase. Gram reaction with 3% KOH and Gram staining also were conducted.

Cultures were maintained both in glycerol medium (4 g nutrient broth, Difco Laboratories; 150 ml glycerol; 500 ml deionized water) in 2-ml cryogenic vials at -70C and subcultured monthly or bimonthly.

Results

Gas chromatographic analysis of fatty acids and supplemental biochemical and staining tests indicated that bacteria associated with S. scapterisci belong to several genera. It is assumed that those bacteria originated from the nematode gut. The ATCC Xenorhabdus strains (19061 and 35271) were used for comparison with the isolates obtained

from S. scapterisci. Isolates obtained from S. carpocapsae also were compared with the ATCC cultures and were used as reference strains. Results obtained from GC analyses as well as from biochemical and Gram tests are presented in Tables 2.1 and 2.2. Additional comments are presented in the report of results for each isolate.

Table 2.1 - Results obtained from gas chromatography analyses of bacteria isolated from *Steinernema scapterisci* and some reference strains.

Isolate	Bacterium species	Similarity index
SS01-1	*Edwardsiela ictulari*	0.379
SS01-2	*E. ictulari*	0.368
SS01-3	*Proteus vulgaris*	0.421
SS01-4	*P. vulgaris*	0.337
SS02-1	*Morganella morganii*	0.588
SS02-2	*M. morganii*	0.619
SS02-3	*M. morganii*	0.619
SS03-1	*M. morganii*	0.872
SS03-2	*Xanthomonas maltophilia*	0.932
SS03-3	*M. morganii*	0.317
SS03-4	*Cedecea davisae*	0.502
SS04-1	*Pseudomonas aureofaciens*	0.845
SS04-2	*P. aureofaciens*	0.879
SS04-3	*P. aureofaciens*	0.902
SS04-4	*P. aureofaciens*	0.890
SS05-1	*Ochrobactrum anthropi*	0.739

Table 2.1 - Continued.

Isolate	Bacterium species	Similarity Index
SS05-2	O. anthropi	0.539
SS05-3a	O. anthropi	0.533
SS05-3b	O. anthropi	0.805
SS05-4	O. anthropi	0.844
SS05-5a	Paracoccus denitrificans	0.129
SS05-5b	O. anthropi	0.716
SC01-1	Moraxella nonliquefaciens	0.260
SC01-2	M. nonliquefaciens	0.426
SC01-3	Xanthomonas maltophilia	0.844
SC01-4	X.maltophilia	0.830
ATCC 35271	M. morganii	0.455
ATCC 35271	Hafnia alvei	0.451
ATCC 35271	H. alvei	0.410
ATCC 19061	Erwinia herbicola	0.822
ATCC 19061	Proteus vulgaris	0.623
ATCC 19061	P. vulgaris	0.751

Table 2.2 - Reaction of bacterial isolates obtained from Sterinernema spp. in biochemical and Gram tests.

Bacterial isolates	Biochemical tests				Gram reaction	
	Hugh - Leifson	KMB fluoresc.	Oxidase	Arginine dihydrol.	KOH	Stain
Xenorhabdus sp.	+	-	-	-	-	-
Xenorhabdus sp.	-	-	+	-	+	-
X. bovienii	+	-	-	-	-	-
Xanthomonas maltophilia	-	-	-	-	-	-
Pseudomonas aureofaciens	-	+	+	+	-	-
P. aureofaciens	-	+	+	+	-	-
Ochrobactrum anthropi	-	-	+	-	+	-
O. anthropi	-	-	+	-	+	-
O. anthropi	-	-	+	-	+	-
Unknown*	-	-	+	-	+	-

Table 2.2 - Continued.

Bacterial isolates	Biochemical tests				Gram reaction	
	Hugh - Leifson	KMB fluoresc.	Oxidase	Arginine dihydrol.	KOH	Stain
Xenorhabdus nematophilus**	+	-	-	-	-	-
Erwinia herbicola***	+	Nt	-	Nt	Nt	-
Pseudomonas putida***	Nt	-	+	-	Nt	Nt
Staphylococcus avenae***	Nt	Nt	Nt	Nt	Nt	+
S. aureus***	Nt	Nt	Nt	Nt	Nt	+
X. maltophilia***	Nt	Nt	Nt	Nt	-	Nt

* - Related to Paracoccus denitrificans.
** - Reference strain, isolated from Steinernema scapterisci.
*** - Reference strain, obtained from the Department of Plant Pathology.
Nt - Not tested.

Two different genera of bacteria were recovered from S. carpocapsae. Isolates SC01-1 and SC01-2 were similar to Xenorhabdus nematophilus (ATCC 19061) as compared in a dendrogram (Euclidian distance 6.04). This was confirmed based upon colony characteristics on T7-TTC medium. Colonies were circular, had irregular margins, and presented a dark blue color, indicating absorbtion of bromothymol blue, a characteristic of the primary phase of Xenorhabdus nematophilus (Akhurst 1980, Akhurst and Boemare 1988) These isolates showed some relationship with Moraxella nonliquefasciens, although the SI were low (0.260 - 0.426). Isolates SC01-3 and SC01-4 matched most closely Xanthomonas maltophilia (SI= 0.830 - 0.844).

Isolates SS01-1, SS01-2, SS01-3, and SS01-4 were obtained from S. scapterisci reared continuously in vivo in the laboratory. Via FA anlysis, these isolates were related remotely to Proteus vulgaris Hauser 1885, and to Edwardsiella ictulari Hawke, McWhorter, Steigerwalt, and Brenner 1981. The low SI (0.304 - 0.358; and 0.305 - 0.379, respectively) implied that these organisms were not in the MIDI reference library. Comparisons with ATCC 19061 (X. nematophilus) and ATCC 35271 (X. bovienii) indicated that the isolates obtained from S. scapterisci are related to those type cultures at the genus level (14.02 - 18.33 Euclidian distance). Moreover, comparisons with the isolate obtained from S. carpocapsae

showed a relationship at the genus level (14.02 Euclidian distance).

Isolates SS02-1, SS02-2, and SS02-3 also were obtained from S. scapterisci maintained in the laboratory through continuous rearing in vivo. FA results showed some relation to Morganella morganii (Winslow, Klinger and Rothberg 1919) Brenner, Farmer, Fanning, Steigerwalt, Klykken, Waten, Hickman, and Ewing 1978, but with a moderate match (0.588 - 0.619). Like the previous isolates, a close match with ATCC 19061 (X. nematophilus), ATCC 35271 (X. bovienii) and X. nematophilus isolated from S. carpocapsae (Euclidian distance 18.33, 10.78, and 14.02, respectively) also was shown indicating that all of them are likely to be the same genus. However, when plated on T7-TTC medium, only red colonies were formed by isolates SS02-1, SS02-2, and SS02-3. It is important to note that these isolates matched with the previous isolates (SS01-1, SS01-2, SS01-3, and SS01-4) at the genus level (18.33 Euclidian distance).

Strains SS03-1, SS03-2, SS03-3, and SS03-4 were isolated from S. scapterisci obtained from infected mole crickets infected at Green Acres Agricultural Farm. FA analyses for SS03-2 resulted in a very high match (0.932) to Xanthomonas maltophilia. Strains SS03-1 and SS03-3 matched M. morganii at distinctly different degrees (0.872 and 0.317, respectively). The first choice match to Isolate SS03-4 was Cedecea davisae Grimont, Grimont, Farmer, and Ashbury 1981 at a low SI

(0.502). However, when these three isolates were compared to the reference species considered in this work, a close match (7.55 Euclidian distance) with ATCC 35271 (X. bovienii) was obtained indicating that these cultures may belong to that species.

Isolates SS04-1, SS04-2, SS04-3, and SS04-4 were obtained from S. scapterisci reared on culture medium. Only one bacterium species was isolated. This was shown to be closely related to Pseudomonas aureofasciens with a high match (0.845 - 0.902). Additional biochemical tests confirmed them to be facultatively anaerobic, Enterobacteriaceae, and in the fluorescent group of Pseudomonas. Variations in colony characters of P. aureofaciens indicate that mutations may occur frequently.

Isolates SS05-1, SS05-2, SS05-3a, SS05-3b, SS05-4, SS05-5a, and SS05-5b were obtained from S. scapterisci collected from natural infestation of mole crickets in LaCrosse, FL. Except for isolate SS05-5a, all of them were shown to be related to Ochrobactrum anthropi Holmes, Popoff, Kiredjian, and Kersters 1988 with SI between 0.533 - 0.844. Isolate SS05-5a was distinct from the rest of the group and was more related to Paracoccus denitrificans (Beijerink 1910) Davis 1969 among the species available in the MIDI system. However, only a low match (0.119 - 0.129) was obtained. Comparisons with all the remaining isolates obtained from S scapterisci as well as with other Xenorhabdus species (ATCC 19061; ATCC

35271; and isolates SC01-1; and SC01-2) showed it belongs to a different genus (66.94 Euclidian distance). This bacterial isolate is a Gram negative coccus, with negative reaction in the Hugh-Leifson, Kings Medium B, and arginine dihydrolase tests. It is oxidase positive (Table 2.2). Although some variation concerning Gram reaction was observed in some of the tests done with KOH, reactions of the various strains in the additional tests were consistent with the characteristics of each one of them (Table 2.2).

Discussion

Many reports in the literature indicate the consistent association of Steinernema spp. with Xenorhabdus spp. (Akhurst 1982, 1983a, 1983b, 1986a, 1986b, Poinar 1979, Thomas and Poinar 1979). The importance of this association, characterized as a mutualistic symbiosis (Poinar, 1979) for both the nematode action in a host, and for the nematode growth in vitro has been demonstrated frequently (Poinar 1966; Akhurst 1980, 1983b; Ehlers et al. 1990). Specificity of Xenorhabdus to species of Steinernema seems to occur (Akhurst 1983b, Dunphy, et al. 1985). Although isolates of bacteria identified as X. bovienii and as Xenorhabdus spp. were obtained from S. scapterisci in the present work, other bacteria belonging to different genera also were isolated. Some of those species have been isolated from Steinernema spp. by other authors. Wieser (1963) reported that Pseudomonas spp. were isolated from S. carpocapsae (DD-136) and were

responsible for the death of the insect host. Poinar (1966) reported that besides Xenorhabdus (=Achromobacter) nematophilus, other bacteria such as Alcaligenes sp., Aerobacter sp., Proteus sp., and Pseudomonas aeruginosa were found to develop with infective juveniles of Steinernema carpocapsae (DD-136) in dead G. mellonella. Poinar indicated that those other species of bacteria were usually present in the intestine of healthy G. mellonella and concluded that they invaded the insect hemocoel after death of the insect. Poinar suggested that although S. carpocapsae acquired cells of those bacteria through indiscriminant feeding, only cells of X. nematophilus remained in the infective juveniles; consequently, only that species was isolated from the nematodes. Lysenko and Weiser (1974) isolated Alcaligenes odorans, Xanthomonas (=Pseudomonas) maltophilia, P. alcaligenes, P. fluorescens, Pseudomonas sp., Acinetobacter sp., Alcaligenes sp., Achromobacter sp. and Flavobacterium sp. from S. carpocapsae. They showed that initially axenic nematodes inoculated with Xanthomonas (=Pseudomonas) maltophilia constituted the most pathogenic complex tested. Association with bacteria other than Xenorhabdus was observed by Boemare et al. (1983) who found Pseudomonas fluorescens and Xanthomonas (=Pseudomonas) maltophilia in addition to other bacteria associated with S. (=Neoaplectana) carpocapsae. Of all the bacteria reported here in association with S. scapterisci, only Ochrobactrum anthropi has not been reported

previously in association with Steinernema spp. This probably is because O. anthropi was described only recently (Holmes et al. 1988). However, several species of Achromobacter and Pseudomonas were synonymmized with O. anthropi. Xenorhabdus nematophilus was originally described as Achromobacter and based on biochemical tests there are similarities between these two genera (Poinar and Thomas 1966). (Pseudomonas spp. have been isolated from S. glaseri (Poinar 1979) and from other closely related nematodes (Anderson and Coleman 1981, Abrams and Mitchell 1978).

The observation of an association of S. scapterisci with various bacterium species is not surprising based on evidence cited by other authors on nematodes in the same or in closely related genera.

Bacteria are the most abundant type of microorganism associated with insects. Associations may be as saprophytes, symbionts or pathogenic agents. The insect pathogenic bacteria may be separated into two groups, one consisting of bacteria which cause disease when they are ingested. Microbes in the other group are ubiquitous and able to cause disease if introduced into the hemolymph, or if the insect host is compromised or stressed. Some of these bacteria, common in the insect gut, may be introduced into the hemocoel by the nematodes during their parasitic action. They may not multiply well in the intestine, and consequently will not produce large populations in that organ, but, if introduced into the

hemocoel, may thrive and cause septicemia. These bacteria are considered potential pathogens (Bucher 1960). A characteristic of a potential pathogen is its ability to multiply in the insect hemolymph. Proteolytic activity of bacteria is strongly correlated with virulence. Xenorhabdus spp. are known to present this characteristic (Kenneth et al. 1990).

It is well known that steinernematid nematodes produce a toxin or factor which affects the insect enzymatic defense response (Burman 1982). Thus, even weak bacterial pathogens may benefit from the association with nematodes. Some bacteria other than Xenorhabdus which were found associated with S. scapterisci are known pathogens of insects (Lyzenko and Weiser 1974). This may explain the relatively easy establishment of S. scapterisci in the field where it has exceeded general expectations in terms of survival under field conditions. It has spread continuously from the original application sites and has been recovered for five years following the first field inoculation (Smart et al., 1991). This nematode species apparently is able to use various bacteria including Xenorhabdus. In this study, infective juveniles were recovered from infected insects while carrying several genera of bacteria. In addition, the results indicate that infective juveniles of S. scapterisci may carry more than one bacterium genus concomitantly.

These results suggest some similarities between S. scapterisci and S. glaseri in terms of specificity of bacterial associations. Poinar (1986) indicated that S. (=Neoaplectana) glaseri, differently from other Steinernema species, has more ability to tolerate and even benefit from other bacteria. Poinar linked this characteristic to a less evolved condition. The Xenorhabdus species associated with S. glaseri does not form the two phases presented by other Xenorhabdus species (Akhurst 1980). Different phases of the Xenorhabdus spp. associated with S. scapterisci in this work were not observed. In addition, the geographical range of S. scapterisci seems to be somewhat similar to that of S. glaseri. It could perhaps be speculated that S. scapterisci is also a less evolved species and in this way, also less specialized.

It is here suggested that S. scapterisci obtained from natural infestations should have its associated bacteria determined and the nematode-bacteria complex possibly catalogued as a strain for future reference. Possible differences in pathogenicity should also be determined.

Additional studies concerning suitability of these bacteria as food sources and reproduction factors for the nematode, as well as the ability of the infective juveniles to retain bacterial cells, is part of another chapter of this dissertation.

CHAPTER 3
DEVELOPMENT AND REPRODUCTION OF STEINERNEMA SCAPTERISCI IN MONOXENIC CULTURES

Introduction

As indicated in Chapter 2, Steinernema scapterisci Nguyen and Smart, 1990 has the ability to use as symbionts various bacterial species which may be present in the soil environment. These bacteria are likely part of the normal microflora of healthy insects or they are present in the rhizosphere of plants where microorganisms and insects usually are abundant. Other species of Steinernema seem to be more restricted in bacterial symbionts since rather specific associations have been determined either from isolations of bacteria from infective juveniles collected in the body of insect hosts or from observations in culture media (Akhurst 1982a, 1983b, 1986a, 1986b, Poinar 1986, Poinar et al. 1988, Thomas and Poinar 1979). Some reports, however, indicate that genera of bacteria other than Xenorhabdus may be associated with steinernematid nematodes. Weiser (1963) reported the occurrence of species of Pseudomonas associated with S. carpocapsae (=Neoaplectana DD-136) and considered them as entomopathogens. Poinar (1966) isolated species of

Pseudomonas, Proteus, Alcaligenes, and Aerobacter from S. carpocapsae but regarded them as non-pathogenic to Galleria mellonella (L.) and indicated that they were usually present in the intestine of healthy G. mellonella. Lysenko and Weiser (1974) also isolated several species of bacteria from S. (=Neoaplectana) carpocapsae and showed that both the bacteria themselves and the bacteria-nematode complexes were pathogenic to G. mellonella. Boemare (1983) and Boemare et al. (1983) reported that not only did they isolate certain bacteria, such as Pseudomonas, Aeromonas, and Enterobacter from S. carpocapsae, but that those bacteria were dominant over others, including X. nematophilus. Nonetheless, they reported that X. nematophilus produces antibiotics that regulate the population of microflora associated with the insect host.

Akhurst (1983b) reported that five species of Steinernema (=Neoaplectana) reproduced on different species of Xenorhabdus. He pointed out that, although each nematode species reproduced best on its own symbiont, the association was not completely specific, and that perhaps more efficient associations could be created.

Associations between steinernematid nematodes and bacteria are of fundamental importance since the bacteria influence nematode growth, reproduction, and survival. There is no nematode growth and reproduction in the absence of bacteria (Poinar and Thomas 1966).

Results reported in Chapter 2 indicate that species of Pseudomonas, Xanthomonas, Ochrobactrum and an unidentified genus related to Paracoccus, in addition to Xenorhabdus spp., are associated with S. scapterisci. The present work investigates the effects of various bacterial species and strains on the development and reproduction of S. scapterisci in monoxenic culture.

Materials and Methods

Before experiments on growth and reproduction of S. scapterisci in monoxenic culture on various bacteria could be conducted, axenic nematodes and bacterial colonies had to be established.

Axenic nematodes

House crickets (Acheta domesticus), obtained at a local bait shop, were exposed to 15,000 to 20,000 infective juveniles of S. scapterisci in petri dishes (100 x 15 mm) lined with two Whatman No. 2 filter papers (Whatman International Ltd., Maidstone, England). Ten house crickets were released in each petri dish which was secured with tape and maintained in an incubator at 25C. Four days after exposure, dead crickets were dissected in sterile saline solution (NaCl 7.5g, KCl 0.35g, $CaCl_2$ 0.21g, deionized water 1,000ml). First-generation female nematodes present in the cadavers were hand-picked and transferred to another dish containing sterile saline solution. The females were rinsed

briefly with a sterilizing solution (0.4 M NaOH, 0.2 M NaOCl; Buecher and Popiel 1989), rinsed again with sterile saline solution, and transferred to a sterile beaker containing the same sterilizing solution as above for 10 minutes and stirred frequently. The solution destroyed the female body tissues and released the eggs. The eggs were rinsed three times with sterile saline solution on a sieve composed of two layers of a 500 mesh nylon cloth (10 μm openings) mounted in a 100 x 15 mm-cylindrical glass device, collected with a sterile pipette and transferred to a sterile glass test tube (150 mm x 20 mm). The average number of eggs in five 10-μl samples was taken to determine the concentration of eggs in the suspension.

Bacterial colonies

Colonies of the following bacteria were obtained from Steinernema scapterisci, from S. carpocapsae, from the American Type Culture Collection (ATCC, Rockville, MD) or from the Plant Pathology Department, University of Florida:

Xenorhabdus sp. (Isolate SS01-1), obtained from S. scapterisci reared continuously in vivo in the Entomology and Nematology Department, University of Florida. This isolate is closely related to X. nematophilus (ATCC culture 19061) and to X. bovienii (ATCC culture 35271) at the genus level.

Xenorhabdus sp. (Isolate SS02-1), same origin as isolate SS01-1. It is closely related to X. nematophilus (ATCC culture 19061) and to X. bovienii (ATCC culture 35271) at the genus

level. It is also related to isolate SS01-1 above but is not the same species.

Xenorhabdus bovienii (Isolate SS03-1), obtained from infective juveniles of S. scapterisci collected from infected mole crickets at the University of Florida Green Acres Agricultural Farm, Alachua County, FL.

Xanthomonas maltophilia (Isolate SS03-2), same origin as isolate SS03-1.

Pseudomonas aureofaciens (Isolate SS04-2), obtained from infective juveniles of S. scapterisci produced in vitro.

Unknown, related to Paracoccus denitrificans (Isolate SS05-5a), obtained from infective juveniles of S. scapterisci collected from mole crickets near LaCrosse, Alachua County, FL. This bacterial isolate is remotely related to Paracoccus denitrificans. It is a Gram negative coccus, with a negative reaction in the Hugh-Leifson, Kings Medium B, and arginine dihydrolase tests and it is oxidase positive.

Ochrobactrum anthropi (Isolate SS05-5b), Same origin as isolate SS05-5a.

Xenorhabdus nematophilus (Isolate SC01-1), obtained from Steinernema carpocapsae strain All, reared in vivo on Galleria mellonella at the Nematology Laboratory, University of Florida.

Xenorhabdus nematophilus (Isolate ATCC 19061) obtained from the American Type Culture Colection.

Xenorhabdus bovienii (Isolate ATCC 35271), same origin as isolate ATCC 19061).

Escherichia coli, obtained from the Plant Pathology Department, University of Florida; culture collection maintained by Dr. R. E. Stall.

Pseudomonas fluorescens Biovar B, same origin as E. coli.

Isolations of bacteria from S. scapterisci and S. carpocapsae were begun by surface sterilizing infective juveniles for 12 hours in an aqueous solution consisting of 0.1% merthiolate and 0.6% streptomycin sulphate for 12 hours. Then the nematodes were rinsed three times with sterile deionized water and macerated in a tissue grinder. The suspension, consisting of nematode tissue and bacterial cells, was plated on Nutrient Agar [8 g Bacto Nutrient Broth (Difco Laboratories, Detroit, MI), 15 g Agar (Fisher Scientific), 1,000 ml deionized water] in petri dishes (100 x 15 mm). These dishes were sealed with Parafilm and maintained in an incubator at 27C. Bacterial colonies were subcultured on the same culture medium to obtain pure cultures. Subcultures grown in Nutrient Broth [8 g Bacto Nutrient Broth (Difco Laboratories), 1,000 ml deionized water) in 250 ml Erlenmeyer flasks under continuous agitation for 24 hours were used in the experiments on development and reproduction of S. scapterisci.

Development and reproduction experiments

Small petri dishes (60 mm x 15 mm) containing brain-heart cholesterol-agar medium [37 g Brain Heart Infusion Dehydrated (Difco Laboratories), 0.02 g cholesterol (Eastman Kodak Co. Rochester, NY), dissolved in 1.0 ml corn oil; 15 g Bacto Agar (Difco Laboratories), 1,000 ml deionized water) were inoculated with a suspension containing 400 to 500 eggs (75 to 100 μl). The dishes were sealed with Parafilm and placed in an incubator (27C) for 24 hours. Those petri dishes which were free from contaminants after 24 hours were inoculated with 100 μl of a 24-hour old bacterial culture.

The life cycle and development of females and males from eggs were determined by observing the cultures under a stereoscopic microscope on a daily basis. The life cycle was considered complete and the number of adult females and males was recorded when egg-bearing females were observed usually 5 days after inoculation. In one of the experiments, reproduction was determined by counting the number of nematodes obtained from the initial adult population 14 days after the eggs were plated on the culture medium. In this instance, nematodes rinsed and collected from the medium surface and the culture medium melted in a water bath at 75C for collecting the remaining nematodes. Counting in both suspensions was done in five 10-μl subsamples in each replication and the average used for the statistical analysis. Three experiments were conducted:

Experiment 1 - Treatments consisted of Xenorhabdus nematophilus (Isolate SC01-1), Pseudomonas aureofaciens (Isolate SS04-2), Unknown (Isolate SS05-5a), Ochrobactrum anthropi (Isolate SS05-3b), and control (no bacteria). Except for the unknown (Isolate SS05-5a) which was replicated three times, all the remaining treatments were replicated four times.

Experiment 2. Treatments were: Xenorhabdus nematophilus (Isolate SC01-1), Xenorhabdus sp. (Isolate , SS01-1), X. bovienii (Isolate SS03-1), Xanthomonas maltophilia (Isolate SS03-2), Pseudomonas aureofaciens (Isolate SS04-2), Ochroactrum anthropi (Isolate SS05-3b), Paracoccus denitrificans (Isolate SS05-5a), Pseudomonas fluorescens Biovar B, Escherichia coli, control 1 (axenic juveniles; no bacteria) and control 2 (axenic juveniles; inactivated cells of Xenorhabdus nematophilus (Isolate SC01-1). The latter was prepared by heating a 24-hour old culture in a water bath at 75C for 30 minutes. Each treatment was replicated five times.

Experiment 3 - Treatments were: X. nematophilus (Isolate SC01-1), Xenorhabdus sp. (Isolate SS02-1), X. bovienii (Isolate SS03-1), Xanthomonas mlatophilia (Isolate SS03-2), Pseudomonas aureofaciens (Isolate SS04-2), Ochrobactrum anthropi (Isolate SS05-3b), Unknown (related to Paracoccus denitrificans (Isolate SS05-5a) Pseudomonas fluorescens, Escherichia coli, and control (axenic nematodes). Each treatment was replicated six times.

All experiments were arranged in a randomized complete block design. Differences in numbers of nematodes produced on the various bacteria were determined by an analysis of variance, and means were separated by Duncan's multiple range test at $P = 0.05$ (Freed et al. 1987)

Results

The procedure for axenization of the eggs used in the three experiments was satisfactory and axenic first-stage juveniles were obtained within 24 hours. Eggs were at different stages of development, including with some first-stage juveniles (Fig. 3.1a). Those juveniles which were released from the eggs during the process of collecting and sterilizing females and eggs were killed by the sterilizing agents. First-stage juveniles exhibited the characteristic enlargement of the proximal portion of the intestine that constitutes the reservoir for bacteria when the juveniles become infective juveniles (Fig. 3.1b).

Experiment 1. No contamination was observed in any of the control plates, nor did any of the axenic nematodes develop to the adult stage. Some juveniles in this treatment remained active for about two weeks but no further development occurred. Some of these juveniles were observed apparently feeding on the remains of dead juveniles (Fig. 3.1c).

The numbers of females and males of Steinernema scapterisci developing after five days in monoxenic cultures with four bacterium species is presented in Table 3.1. All

Figure 3.1 Steinernema scapterisci on brain-heart cholesterol agar: a) Eggs at different stages of development, including first stage juvenile (arrow). Bar = 15μm. b) First stage juvenile showing enlargement of the proximal portion of the intestine which constitutes the reservoir for bacteria when the juveniles become infective juveniles. Bar = 130 μm. c) Axenic second stage juvenile apparently feeding on the remains of a dead juvenile in culture. Bar = 130 μm.

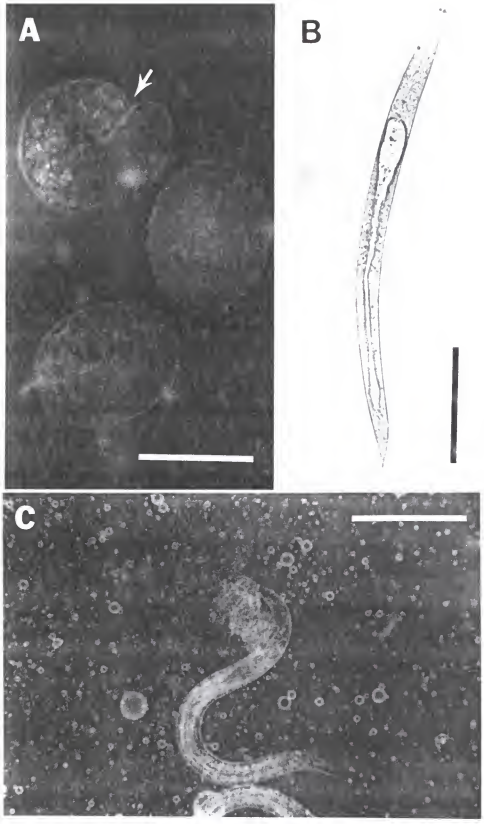
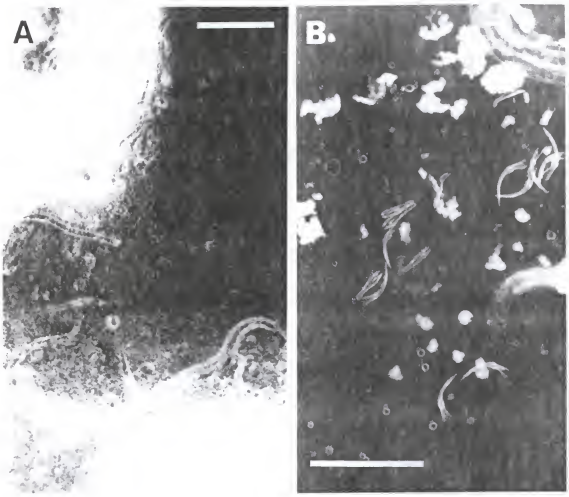

A
B
C

Figure 3.2. Steinernema scapterisci in monoxenic culture with Xenorhabdus nematophilus on brain-heart cholesterol agar. a) Juveniles and eggs 4 days after axenic eggs were plated and 3 days after bacteria were inoculated. Bar = 250 μm. b) Spicules and gubernacula on the medium surface after males died and decomposed. Bar = 158 μm.


A
B.

bacteria used in this experiment served as food for S. scapterisci. Development of first-stage juveniles to adult males and females in all treatments inoculated with bacteria occurred in four to five days after the eggs were plated (Fig. 3.2a). On all bacteria, males appeared one day earlier than females. Development of nematodes was quite uniform in all four monoxenic cultures; between 8.7% and 11.6% of the eggs hatched and developed to adults. There were no differences ($P = 0.05$) in numbers of adults obtained in the four treatments involving bacteria. Thus, Xenorhabdus nematophilus, which was isolated from S. carpocapsae, was as suitable for growth and reproduction of S. scapterisci as were the other bacteria, which were isolated from S. scapterisci.

As the population increased, juveniles migrated to the lid of the petri dishes, where they formed isolated groupings and many streams of migrating columns. These columns were composed mainly of third-stage infective juveniles. Nematode activity decreased but did not cease after the nematodes formed compact columns (Figs. 3.3).

Polyhedral crystals on the culture medium were observed in all cultures. These probably were metabolic by-products of the bacteria. Also, after the males died and disintegrated, the spicules and gubernacula remained intact on the medium surface. These organs were observed in high numbers spread throughout all the petri dishes (Fig. 3.2b). No differences were noted in the degree of occurrence of this phenomenom in

the various monoxenic cultures were noted. Females had large numbers of bacterial cells in the intestine including a few in the rectum. At least most of these cells apparently were intact. Cells were seen also in the lumen of the esophagus (Fig. 3.4a, b) and these had the same appearance as those in the intestine. Interestingly, bacterial cells also were observed in the tissues outside the esophagus of both juveniles and adults (Fig. 3.4c).

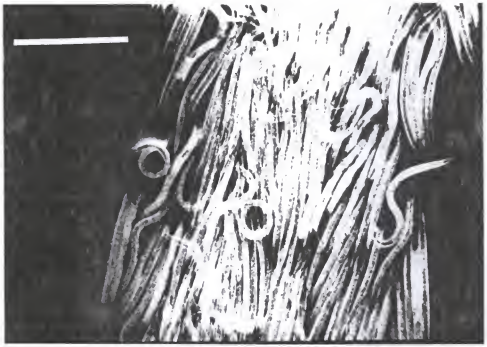


Figure 3.3. Migratory columm composed by Steinernema scapterisci infective juveniles in monoxenic culture with Xenorhabdus nematophilus. Bar = 389 μm.

Figure 3.4. Steinernema scapterisci in monoxenic culture on brain-heart cholesterol agar. a) Anterior end of female with bacterial cells in the lumen of the esophagus. b) Anterior end of female showing bacterial cells in the buccal cavity (arrow) and in the lumen of the esophagus. c) Basal portion of the esophagus of a female showing bacterial cells outside the esophagus. Bar = 15 μm, all same scale.

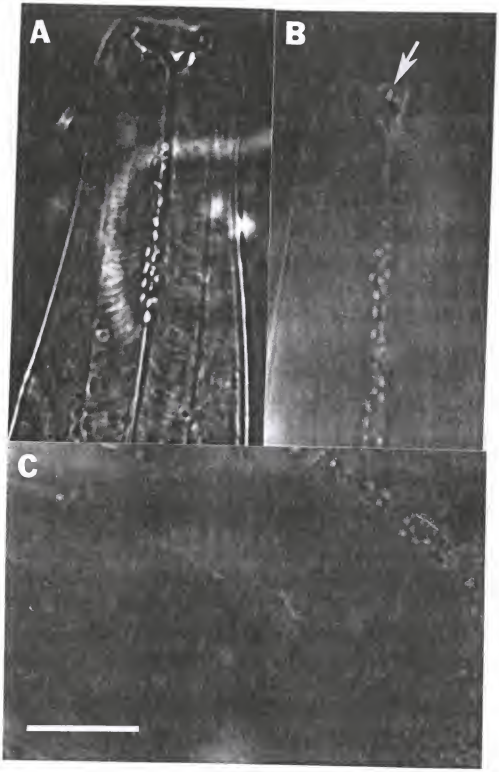

A
B
C

Table 3.1. Number of adults of Steinernema scapterisci obtained from 400 - 500 axeb=nic eggs in monoxenic cultures with bacteria five days after eggs were placed on the culture medium.

Treatment	Number of adult nematodes/petri dish		
	Females	Males	Total
Xenorhabdus nematophilus	29.0 ± 11.7	18.2 ± 5.0	47.5 ± 15.7
Pseudomonas aureofaciens	28.2 ± 14.8	23.7 ± 15.7	52.0 ± 30.5
Ochrobactrum anthropi	30.0 ± 12.3	13.5 ± 2.5	43.5 ± 13.1
Unknown*	30.3 ± 1.2	27.3 ± 2.9	58.0 ± 1.4
Control (axenic nematodes)	Nd**	Nd	Nd

Data are means ± standard deviations of four replications, except for the unknown bacterium which had three replications. No significant ($P = 0.05$) differences were observed.
* - Related to Paracoccus denitrificans.
** - No development to adult stage.

Experiment 2. The number of adults developing from eggs was low, ranging from 0.2% to 14.2%. No contamination was observed in the control plates throughout the experiment. The axenic juveniles in these treatments survived for at least two weeks and developed to second and third stage but did not reach the adult stage. Nematodes developed in all the treatments containing live bacteria, but differences occurred in the number of individuals reaching the adult stage (Table 3.2). The bacteria isolated from the nematodes themselves as well as some foreign bacteria served as food for the nematodes and induced their growth. Some of the bacterium species, especially Pseudomonas fluorescens, developed a very dense growth but apparently this was not detrimental to the activity and development of the nematodes. At about six days after inoculation, the nematodes had consumed many of the bacterial cells and consequently the bacterial growth was less dense.

Most of the bacterial species tested were suitable for reproduction of S. scapterisci based on numbers obtained 14 days after inoculation. The exception was Xenorhabdus sp., (Isolate SS01-1) which induced initial growth but only poor reproduction with no further development of the population (data not shown or analyzed). The other bacterial isolates were subsequently very efficient, resulting in high ratios of Pf/Pi, representing population increases >400-fold. A greater rate of reproduction (Pf/Pi) of S.scapterisci occurred on

Xenorhabdus nematophilus and Pseudomonas aureofaciens than on five of the other bacterial isolates (Table 3.3).

Table 3.2. Number of adult Steinernema scapterisci obtained from 400 - 500 axenic eggs in monoxenic cultures with bacteria five days after eggs were inoculated on the culture medium.

Treatments	Number of adult nematodes/petri dish		
	Females	Males	Total
Pseudomonas fluorescens	26.8 ± 11.3 a	18.3 ± 8.8 a	45.2 ± 18.2 a
Ochrobactrum anthropi	25.5 ± 9.8 a	17.0 ± 7.6 a	42.5 ± 15.4 a
Xanthomonas maltophilia	22.3 ± 8.6 a	17.3 ± 5.9 a	39.7 ± 14.1 a
Xenorhabdus nematophilus	17.3 ± 10.0 ab	15.2 ± 9.9 ab	32.5 ± 19.8 ab
Escherichia coli	8.33 ± 3.3 bc	10.8 ± 5.5 abc	19.2 ± 8.4 bc
Unknown	11.7 ± 5.8 bc	7.3 ± 3.5 bc	19.0 ± 7.9 bc
Pseudomonas aureofaciens	4.3 ± 3.3 c	4.3 ± 4.1 c	8.7 ± 5.8 c
Xenorhabdus bovienii	3.2 ± 2.8 c	4.0 ± 2.5 c	7.2 ± 5.0 c
Xenorhabdus sp.	2.5 ± 2.5 c	2.8 ± 3.0 c	5.3 ± 3.3 c

Table 3.2. Continued.

Treatments	Number of adult nematodes/petri dish		
	Females	Males	Total
Control 1	Nd*	Nd	Nd
Control 2	Nd	Nd	Nd

Data are means ± standard deviation of five replications. Means in columns followed by same letter are not different (P = 0.05) according to Duncan's Multiple Range Test.
Control 1 = No bacteria (axenic nematodes).
Control 2 = Inactivated cells of Xenorhabdus nematophilus (isolate SC01-1).
* = No development.

Table 3.3. Number of Steinernema scapterisci from 400 - 500 axenic eggs in monoxenic cultures with bacteria 14 days after eggs were inoculated on the culture medium.

Treatment	Number of nematodes/petri dish		
	Initial population*	Final population**	Pf/Pi**
Pseudomonas aureofaciens	7 ± 6.2 d	43,574 ± 30,948.1 b	6,491 ± 5,201.8 a
Xenorhabdus nematophilus	30 ± 23.2 abc	129,602 ± 28,640.4 a	5,956 ± 2,691.4 a
Pseudomonas fluorescens	45 ± 22.4 a	117,100 ± 25,096.3 a	3,949 ± 3,847.1 ab
Unknown†	21 ± 7.1 bcd	36,819 ± 13,014.0 b	1,821 ± 758.6 b
Xenorhabdus bovienii	5 ± 3.9 d	10,521 ± 16,900.9 c	1,280 ± 1,557.8 b
Escherichia coli	18 ± 9.6 cd	15,702 ± 19,385.0 bc	857 ± 1,062.4 b
Ochrobactrum anthropi	42 ± 18.8 ab	19,223 ± 17,222.2 bc	446 ± 323.4 b
Xanthomonas maltophilia	38 ± 16.8 abc	15,689 ± 5,736.0 bc	433 ± 82.2 b

Table 3.3. Continued.

Treatment	Number of nematodes/petri dish		
	Initial population*	Final population**	Pf/Pi***
Control 1	Nd****	Nd	Nd
Control 2	Nd	Nd	Nd

Data are means of five replications. Means in columns followed by same letter are not different (P = 0.05) according to Duncan's Multiple Range Test.
† - Isolate SS05-5a, related to Paracoccus denitrificans.
* - Initial population measured five days after eggs were inoculated on culture medium.
** - Fourteen days after eggs were inoculated on culture medium.
*** - Population at 14 days after eggs were inoculated on culture medium divided by population five days after eggs were inoculated on culture medium.
**** - No development to adult stage.

Experiment 3. Five days after eggs were plated, the greatest number of females developed on Pseudomonas fluorescens, Xanthomonas maltophilia, and Ochrobactrum anthropi (Table 3.4). In general, the trend in suitability among bacteria for nematode development was the same as in Experiment 2, except that no bacteria and also no nematodes developed in five of six replications of the treatment with Xenorhabdus nematophilus.

Table 3.4. Number of *Steinernema scapterisci* from 400 - 500 axenic eggs in monoxenic culture with bacteria five days after eggs were plated on culture meduim.

Treatment	Number of nematodes/petri dish		
	Females	Males	Total
Pseudomonas fluorescens	14 ± 8.4 a	5.2 ± 2.5 bc	19.2 ± 9.8 a
Xanthomonas maltophilia	8.5 ± 5.6 a	9.7 ± 3.4 a	18.2 ± 6.9 ab
Ochrobactrum anthropi	8.5 ± 5.3 a	7.2 ± 3.6 ab	15.7 ± 7.3 ab
Escherichia coli	6.7 ± 3.1 bc	5.5 ± 2.6 bc	10.7 ± 4.7 abc
Xenorhabdus sp.	4.5 ± 2.2 bc	5.0 ± 1.4 bc	9.5 ± 3.2 bc
Unknown†	4.2 ± 4.0 bc	5.2 ± 3.3 bc	9.3 ± 6.9 bc

Table 3.4. Continued.

Treatment	Number of nematodes/petri dish		
	Females	Males	Total
P. aureofaciens	1.2 ± 1.1 c	1.7 ± 3.7 c	3.3 ± 7.4 c
Xenorhabdus nematophilus	1.7 ± 3.7 c	1.7 ± 3.7 c	3.3 ± 7.4 c
X. bovienii	Nd	Nd	Nd
Control	Nd	Nd	Nd

Data are means ± standard deviations of five replications. Means in columns followed by same letter are not different (P = 0.05) according to Duncan's Multiple range Test.
† - Unknown, isolate SS05-5a (related to Paracoccus denitrificans.

Discussion

Based on the three experiments reported in this chapter, it can be concluded that all the bacterial species served as a food source and promoted growth of S. scapterisci, but some were more suitable than others. In Experiment 1, bacteria obtained from S. carpocapsae All strain was as suitable for growth of S. scapterisci as bacteria found associated with this nematode species. In Experiment 2, the best associations in decreasing order were Pseudomonas fluorescens, Ochrobactrum antropi, Xanthomonas maltophilia, and Xenorhabdus nematophilus.

Steinernema scapterisci did not develop when inoculated with inactivated cells of X. nematophilus. The nematode requires active bacterial cells for its growth and reproduction. This is contrary to what has been observed for S. carpocapsae and S. glaseri (Glaser 1940, Glaser et al. 1942, Stoll 1959, Boemare 1983, Boemare et al. 1983).

Nematode reproduction evaluated 14 days after eggs were inoculated onto the culture medium was highest in monoxenic cultures of Xenorhabdus nematophilus and Pseudomonas fluorescens. The former species was obtained from S.carpocapsae strain All and the latter is a soil and water microorganism which can be a saprophyte or an oportunistic animal pathogen.

The results of these tests indicate that S. scapterisci is able to live and reproduce on bacterial symbionts of other Steinernema species, on its own symbionts, and even on non-symbionts. Akhurst (1983b) observed that five different species of Steinernema reproduced on Xenorhabdus symbionts of any other species of the nematode, but retention of bacteria by infective juveniles was lower in associations of nematodes with symbionts of other Steinernema species than in associations with its own symbiont. This behavior indicates some specificity. This was not observed to occur with S. scapterisci. Except for one isolate, Xenorhabdus bovienii (Isolate SS03-1), in Experiment 3, all other bacterial isolates with varying degrees were suitable for nematode reproduction. Also in Experiment 3, in plates inoculted with Xenorhabdus nematophilus (Isolate SC01-1), only one out of six replications showed bacterial growth. No nematode growth and reproduction in those replications without bacterial growth. Xenorhabdus is defined as a genus of bacteria with low plating efficiency (Brenner 1985) and this may be one reason for the lack of nematode growth when inoculated with that isolate. Another possible explanation would be phase variation of the bacteria (Akhurst 1980). It was not possible, however, to speculate here about occurrence of primary and secondary phases of the bacteria, since colony characteristics were those of the primary phase.

Steinernematid nematodes have been reported in association with bacteria other than Xenorhabdus by other authors. (Boemare et al. 1983, Ehlers et al. 1990, Lyzenko and Weiser 1974, Poinar 1966) The life cycle of S. carpocapsae, however, was delayed by six days in monoxenic cultures with E. coli (Ehlers et al. 1990). In the present study, such an extended delay did not occur when axenic S. scapterisci were cultured on E. coli. The nematode completed its life cycle in five days - one day later than on the other monoxenic cultures - and reproduced at ratios similar to those on bacteria normally isolated from this nematode species.

The life cycle of S. scapterisci was completed almost simultaneously in cultures with all the bacteria and was shorter than in vivo cultures. Nguyen and Smart (1991) observed that in the absence of an adequate food supply, at 24C, it takes six to seven days for S. scapterisci to complete its life cycle on host tissues. The temperature, however, was lower than the temperature in which the experiments reported here (27C) were conducted.

These results, suggest that the bacterial associations reported in Chapter 2 may occur in nature and that the nematode may thrive in the presence of various bacterium species. This lack of selectivity may be an indication of a less evolved species when compared to other Steinernema species but it may be on the other hand, an advantageous maneuver that permits the nematode to survive in nature. In

fact, the easy establishment of S. scapterisci in Florida after its introduction from South America (Smart et al. 1991) seems to corroborate this hypothesis.

CHAPTER 4
CHARACTERISTICS OF ASSOCIATIONS BETWEEN *STEINERNEMA SCAPTERISCI* AND VARIOUS BACTERIA IN MONOXENIC CULTURES

Introduction

Nematodes in the genus *Steinernema* Travassos, 1927 are known to live in association with bacterial symbionts which are carried by the nematode infective juveniles (Poinar 1966, Thomas and Poinar 1979, Akhurst 1983a, 1986a, 1986b). In most cases, there seems to be a rather specific symbiosis, in which the nematodes enable the bacteria to reach a potential insect host and the bacteria, in turn, kill the insect and provide a source of food for growth and reproduction of the nematode (Akhurst 1983b, Dunphy et al. 1985). While various genera of bacteria may be associated with *S. carpocapsae*, the association with *Xenorhabdus* seems to be constant and stable for at least most of the *Steinernema* species. Poinar (1966) reported that *S. carpocapsae* can be associated with *Pseudomonas aeruginosa*, *Proteus* sp., *Alcaligenes* sp., and *Aerobacter* sp., in addition to *Xenorhabdus nematophilus* but only cells of the latter were isolated from infective juveniles, suggesting that only *X.nematophilus* was retained by this nematode species. Poinar concluded that oportunistic

bacteria were eliminated during the transformation of third-stage juveniles to infective juveniles. Boemare et al. (1983), demonstrated that S. carpocapsae can utilize Enterobacter agglomerans, Serratia liquefaciens, and Pseudomonas fluorescens as well as X. nematophilus and that these associations are maintained as demonstrated by infectivity tests.

Steinernema scapterisci introduced from Uruguay in 1985 is an efficient biological control agent of the mole crickets Scapteriscus borellii and S. vicinus in Florida (Smart et al. 1990). Isolation and characterization of bacteria associated with this nematode species were performed earlier but no attempt to identify them was done (Nguyen 1988).

As part of the present work, isolations and identification of bacteria associated with infective juveniles of S. scapterisci indicated that in nature these nematodes may become associated with more than one species of Xenorhabdus as well as with other genera of bacteria (Chapter 2). In culture, S. scapterisci in culture is not selective concerning bacterial symbionts and can feed and reproduce not only on cultures of bacteria typically found in association with this species, but can also use X. nematophilus, the symbiont of S. carpocapsae, and bacteria from other sources as efficiently as its own symbionts (Chapter 3).

The objectives of this work were to determine if infective juveniles of S. scapterisci would retain cells of

various bacterial species; to determine if these bacterial species can be pathogenic to insects; and to determine if infective juveniles produced in monoxenic cultures with various bacteria are infective to Scapteriscus borellii.

Materials and Methods

Bacteria retention

The bacterial isolates used in this work were obtained from various sources and identified as described in Chapter 1. Axenic first-stage nematodes and bacterial cultures were obtained and inoculated as described in Chapter 3, except that in this study they were produced both in 60 mm petri dishes and in 150 x 15 mm test tubes. About two to three weeks after inoculation, infective juveniles were extracted from the medium and stained (Esser 1973, modified by Esser, pers. comm.) in order to allow the visualization of bacterial cells inside the nematode infective juveniles. The procedure consisted of transferring the infective juveniles to a syracuse watch glass (18 mm internal diameter) and adding 5 drops of iodine. After one minute, the suspension was heated for 2.45 minutes at 35C on a hot plate. Immediately after heating, 1.7 ml of lactophenol were added and the suspension reheated for one minute at 32C. The hot plate was turned off and the suspension was left on the hot plate and allowed to cool for about 20 minutes. Nematodes were mounted on glass slides in the same fixing solution, and examined under a

compound microscope. The number of cells in the proximal portion of the intestine was determined.

The treatments consisted of infective juveniles of S. scapterisci reared monoxenically on brain-heart-cholesterol agar [37 g dehydrated brain heart infusion (Difco Laboratories, Detroit, Michigan), 15 g agar, Fisher Scientific, Fair Lawn, NJ), 0.02 g cholesterol (Eastman Kodak Co. Rochester, NY), 1,000 ml deionized water) in 150 x 15 mm test tubes, at 27C. Axenic nematodes were inoculated according to procedure described in Chapter 3, with the following bacterial species:

1. Xenorhabdus nematophilus (Isolate SC01-1) obtained from S. carpocapsae strain All (Entomology and Nematology Department, University of Florida).

2. Pseudomonas aureofaciens (SS04-2), isolated from S. scapterisci reared in culture medium (Entomology and Nematology Department, University of Florida).

3. Xanthomonas maltophilia (SS03-2) isolated from S. scapterisci collected originally at the Green Acres Agronomy Research Farm, University of Florida, Alachua, FL. and reared in vivo on house crickets (Acheta domesticus) and mole crickets (Scapteriscus spp.).

4. Ochrobactrum anthropi (SS05-3b) isolated from S. scapterisci collected originally at LaCrosse, Alachua, FL, and reared on the same hosts as No. 3.

5. Unknown, related to Paracoccus denitrificans (SS05-5a) obtained from S. scapterisci collected at LaCrosse, Alachua, FL, and reared on the same hosts as No. 3.

6. Control - this treatment consisted of nematodes reared in vivo in contrast to the other treatments which consisted of nematodes produced in monoxenic cultures. The nematodes were collected at LaCrosse, Alachua, FL and populations were increased on the same hosts as in No. 3.

Data consisting of the number of bacterial cells per infective juvenile were submitted to a one-way analysis of variance using the MSTAT Program (Freed et al. 1987). Mean separation among the six bacterial species was performed by Duncan's Multiple Range Test.

Pathogenicity of bacteria

Bacterial isolates were obtained from S. scapterisci from various sources and identified as described in Chapter 2. Cultures 24 hours old were used to prepare suspensions for inoculation. The concentration of cells in the suspension was determined in a Bausch & Lomb Spectronic 21 spectrophotometer (Rochester, NY) and adjusted to give a concentration of 10^8 cells/ml. The insects were anesthetized initially with CO_2 for a few minutes and then inoculated with 10 μl of the bacterial suspension. A hypodermic syringe was used in Experiments 1 and 2, and a Hamilton microliter syringe (Hamilton Co., Reno, NE) in Experiment 3. The needle was inserted approximately 2 mm deep laterally in the anterior portion of the thorax.

Inoculated insects were maintained individually in 5.4-cm long x 2.7-cm diameter snap-cap plastic vials at 25C. The experimental design was a randomized complete block with a variable number of replications, as specified for each of the three experiments reported here. Mortality was assessed at 24 and 48 hours after inoculation.

Experiment 1: Treatments were: Xenorhabdus sp. (Isolate SS01-1), Xenorhabdus sp. (Isolate SS01-2), Xenorhabdus sp. (Isolate SS02-1), X. bovienii (Isolate SS03-1), Xanthomonas maltophilia (Isolate SS03-1), and control (saline solution: 7.5 g NaCl, 0.35 g KCl, 0.21 g $CaCl_2$, 1,000 ml deionized water). The insect species tested was Scapteriscus borellii with 3 replications composed of 4 insects each per treatment.

Experiment 2 - Treatments were: Xenorhabdus sp. (Isolate SS02-1), Xenorhabdus sp. (Isolate SS02-2), X. bovienii (Isolate SS03-1), Pseudomonas aureofaciens (Isolates SS04-1, SS04-2, SS04-3, SS04-4) and control (saline solution, as in Experiment 1). The insect species tested was Acheta domesticus with 3 replications composed of 4 insects each per treatment.

Experiment 3 - Treatments were: X. nematophilus (Isolate SC01-1), and X. bovienii (Isolate SS03-1). Each bacterium species was inoculated at 3 initial doses, with 3 replications of 5 insects each. A control treatment composed of the same number of replications and consisting of inoculation of saline solution (as in Experiment 1), was added to each bacterium species. The insect species tested was A. domesticus.

Data from Experiments 1 and 2 were submitted to a one-way analysis of variance (ANOVA) and the means separated by Duncan's Multiple Range Test, while a factorial ANOVA was used for Experiment 3 (Freed et al. 1987).

Pathogenicity of infective juveniles from monoxenic cultures

Infective juveniles of S. scapterisci were obtained in monoxenic cultures following the procedure described in Chapter 3. The cultures, however, were all established in 150 x 15 mm test tubes with brain-heart-cholesterol agar [37 g brain-heart infusion dehydrated (Difco Laboratories), 0.02 g cholesterol (Eastman Kodak) 15 g agar (Fisher Scientific) 1,000 ml deionized water]. Cultures were maintained in an incubator at 25C. Infective juveniles were extracted from the medium by washing with sterile deionized water and the concentration determined by counting five subsamples from each inoculum. The cultures used in this experiment consisted of S. scapterisci infective juveniles associated with the following bacterial isolates: Ochrobactrum anthropi (Isolate SS05-3b); Pseudomonas aureofaciens (Isolate SS04-2); Xenorhabdus nematophilus (Isolate SC01-1); Unknown, related to Paracoccus denitrificans (Isolate SS05-5a); Two types of controls were used in this experiment. The positive control consisted of infective juveniles from in vivo cultures. The negative control consisted only of deionized water. The inoculum suspension was calibrated to give a dose of 5,000 infective juveniles in 1 ml of deionized water. The inoculum was

transferred via an automatic pipette to 9 g of a sandy soil (98% sand; 0 % silt; 2% clay) in 5.4-cm long x 2.7-cm diameter snap cap plastic vials. One adult of Scapteriscus borellii was placed in each vial. Vials were maintained in an incubator at 25C, and the treatments arranged in a randomized complete block design. Mortality was assessed at 24, 48, 72, and 96 hours after inoculation, and the percentage of dead insects with each bacterial isolate were compared by an Analysis of Variance.

Results

Bacterial retention

Bacterial cells were present throughout the intestine in all of the associations tested. In all of the juveniles examined, however, most of these cells were concentrated in a relatively small, round vesicle in the anterior portion of the intestine. All (100%) infective juveniles examined in all the associations retained bacteria (Table 4.1). Infective juveniles inoculated with Ochrobactrum anthropi retained significantly more bacterial cells than in the other associations but juveniles in association with Xanthomonas maltophilia retained significantly less bacteria than most of the other associations.

Pathogenicity of bacteria

Experiment 1. At 24 hours after inoculation, mortality of Scapteriscus borellii was significantly greater with

Xenorhabdus bovienii and Xanthomonas maltophilia than with all other bacterial isolates or the control (Table 4.2). At 48 hours, mortality with every isolate of bacteria was significantly different from the control, indicating that all the bacteria tested were pathogenic.

Experiment 2. At 24 hours after inoculation, host mortality from every isolate was similar ($P = 0.05$) to that of the control (Table 4.3). At 48 hours, only one isolate of P. aureofaciens isolate was different ($P = 0.05$) from the control. However, mortality caused by E. coli, Xenorhabdus sp. and X. bovienii was not different from that caused by P. aureofaciens.

Experiment 3: At 24 hours, both the bacterial species and the inoculum level had highly significant ($P = 0.001$) effects on mortality of A. domesticus (Table 4.4). In addition, the interaction of bacterial species x inoculum level on A. domesticus mortality was highly significant ($P = 0.001$). This interaction likely results from the increased mortality from the 10^6 dosage of X. nematophilus compared to the 10^6 dosage of X. bovienii. At 48 hours, only inoculum level had a significant ($P = 0.05$) effect on A. domesticus mortality. However, the increased mortality resulting from X. nematophilus inoculation compared to X. bovienii was significant at $P = 0.10$. No significant ($P = 0.10$) bacterium species x inoculum level interaction was observed.

Table 4.1 - Bacterial retention by 50 randomly selected infective juveniles of *Steinernema scapterisci* in monoxenic cultures.

Bacterial isolates	Frequency in IJ (%)	Cells/IJ	
		Mean ± SD*	Range
Ochrobactrum anthropi	100	35.1 ± 10.6 a	11 - 61
Unknown (rel. to *Paracoccus denitrificans*)	100	27.5 ± 10.4 b	11 - 49
In vivo culture**	100	27.3 ± 9.5 b	10 - 53
Xenorhabdus nematophilus	100	26.4 ± 10.3 b	12 - 60
Pseudomonas aureofaciens	100	22.8 ± 9.4 bc	10 - 46
Xanthomonas maltophilia	100	19.6 ± 8.2 c	5 - 40

* Means in columns followed by the same letter are not significantly different ($P = 0.05$).
** Infective juveniles originated from nematodes collected at LaCrosse, Alachua County.

Table 4.2 - Percent mortality of Scapteriscus borellii by bacterial symbionts of Steinernema scapterisci. Experiment 1.

Bacterial isolates	Hours after inoculation	
	24	48
Xenorhabdus bovienii	91.7 a	100.0 a
Xanthomonas maltophilia	83.3 a	91.7 a
Xenorhabdus sp.	33.3 b	91.7 a
Xenorhabdus sp.	16.7 b	66.7 ab
Xenorhabdus sp.	16.7 b	50.0 b
Control (Saline solution)	8.3 b	8.3 c

* Means in columns followed by the same letter are not significantly different ($P = 0.05$).

Table 4.3 - Percent mortality of Acheta domesticus by various species of bacteria. Experiment 2.*

Bacterial isolates	Hours after the inoculation	
	24	48
Pseudomonas aureofaciens	16.7 ab	83.3 a
Escherichia coli	16.7 ab	66.7 ab
Xenorhabdus sp.	33.3 a	50.0 abc
X. bovienii	8.3 ab	50.0 abc
P. aureofaciens	16.7 ab	33.3 bc
P. aureofaciens	8.3 ab	33.3 bc
Xenorhabdus sp.	16.7 ab	25.0 bc
Control (Saline solution)	16.7 ab	25.0 bc
P. aureofaciens	0.0 b	8.33 c

* - Means in columns followed by the same letter are not significantly different (P = 0.05).

Table 4.4 - Percent mortality of Acheta domesticus from Xenorhabdus spp. at four inoculation levels. Experiment 3.

Bacterial isolates	Inoculum levels*	Hours after inoc.	
		24	48
Xenorhabdus bovienii	0	6.7	20.0
	100	0.0	13.3
	10,000	0.0	6.7
	1,000,000	20.0	46.7
Xenorhabdus nematophilus	0	0.0	20.0
	100	6.7	33.3
	10,000	0.0	6.7
	1,000,000	80.0	93.3

* - Number of bacterial cells/insect.

Pathogenicity of infective juveniles from monoxenic cultures:

All the combinations of nematodes and bacteria were successful in infecting adults of Scapteriscus borellii (Table 4.5). Ochrobactrum anthropi was the only isolate showing mortality higher than the control at 24 hours. At $\geq$ 48 hours after inoculation, all nematode and bacterial complexes from monoxenic cultures were equally pathogenic and comparable to nematodes from in vivo cultures.

Table 4.5. Percent mortality of *Scapteriscus borellii* by infective juveniles of *Steinernema scapterisci* associated with various bacterium species.

Bacterial species in complex	Hours after inoculation*			
	24	48	76	92
O. anthropi	37.50 a	68.75 a	75.00 a	81.25 a
P. aureofaciens	18.75 ab	68.75 a	81.25 a	81.25 a
X. nematophilus	0.00 b	56.25 a	75.00 a	81.25 a
Unknown†	18.75 ab	50.00 a	68.75 a	75.00 a
Mixed bacteria**	25.00 ab	56.25 a	62.50 a	62.50 a
Control***	0.00 b	0.00 b	0.00 b	0.00 b

† Related to *Paracoccus denitrificans*.
* Means in columns followed by the same letter are not significantly different ($P = 0.05$).
** Infective juveniles from *in vivo* culture from which *O. anthropi* and an unknown genus of bacteria (related to *P. denitrificans*) were isolated.
*** Deionized water.

Discussion

All the bacterial species tested were retained in the anterior portion of the intestine of infective juveniles of S. scapterisci. In all the associations, all infective juveniles examined retained bacterial cells. The number of cells retained, however, was significantly different in some of these complexes.

While many cells were scattered throughout the intestine in all the associations with bacteria, only those cells present in the anterior portion of the intestine were considered as retained by infective juveniles. In this area of the intestine, most of the bacterial cells are retained in a specialized circular structure posterior to the basal bulb referred to as a vesicle.

Akhurst (1983b) demonstrated that while 94%, 88% and 83% of infective juveniles, respectively, of the species S.feltiae (=Neoaplectana bibionis) , S. carpocapsae (=Neoaplectana feltiae) and S. kraussei retained bacteria, only 49% of S. (=Neoaplectana) glaseri infective juveniles did so. Bird and Akhurst (1983) observed that bacteria in S. feltiae (=bibionis and S. kraussei are tightly packed inside the vesicle while they are rather loose in S. glaseri. These authors suggested that although species of Steinernema (=Neoaplectana) were able to feed and grow on symbionts of other nematodes in the same

genus, only cells of specific symbionts were retained; all strains of Xenorhabdus, which originated from other species were eliminated. Poinar (1966) reported that the number of cells retained by infective juveniles of S. carpocapsae (= Neoaplectana DD-136 strain) infective juveniles ranged between 10 and 200 per nematode, which compares favorably with the range of 5 - 61 cells per infective juvenile of S. scapterisci reported here (Table 4.1).

The present observations supplement other observations reported in Chapters 2 and 3 concerning bacteria associations. Steinernema scapterisci was shown to acquire cells of various bacterial species, either in nature or when inoculated in axenic nematodes. These cells are not eliminated and although there were some statistical differences in relation to numbers of cells retained, all the bacterial isolates tested were retained by the infective juveniles suggesting that these associations might persist in natural conditions. No attempts, however, were made to determine behavior in the presence of polyxenic associations.

These observations demonstrate that S. scapterisci shows distinct characteristics when compared to other Steinernema species which are known to be more specific. Poinar (1966) has suggested that, although S. carpocapsae (DD-136) can be associated with other bacteria in addition to X. nematophilus, infective juveniles retain solely cells of the latter. Boemare (1983), however, demonstrated that S. (= Neoaplectana)

caprpocapsae DD-136 strain is able to feed and reproduce on several other bacterial genera and that juveniles obtained from such associations are infective and cause the death of Galleria mellonella.

Most of the bacteria found associated with S. scapterisci were pathogenic to either Scapteriscus borellii or Acheta domesticus. Xenorhabdus spp. were generally pathogenic, with some variation between experiments, perhaps due to a more prolonged period of subculture of some isolates. Possible modification from the primary phase to secondary phase can be detected on differential culture medium for most of the Xenorhabdus species (Akhurst 1980). No differences in color of the colonies of the isolates used in this work were observed on this medium. Thus, it is not known if changes concerning phases of the bacteria occurred that might have caused differences in pathogenicity. The bacterium Xenorhabdus poinarii is known to be non-pathogenic to Galleria mellonella, although infective juveniles of S. glaseri carrying this same bacterium species are highly pathogenic to that insect (Akhurst 1986a). A similar situation might be the case in our studies.

Differences were observed also among isolates of P. aureofaciens. Although these isolates were all the same age, variations of colony characteristics on Nutrient Agar were observed (Dr. R. E. Stall, pers. comm.), and possibly these

might be different strains. No attempt to further characterize these isolates was done.

Xanthomonas maltophilia, isolated from S. scapterisci, was pathogenic to Scapteriscus borellii. This bacterium species is a common soil microorganism which is not usually an insect pathogen. However, non-pathogens can actually cause disease and frequently insect death when they are introduced into the hemocoel (Poinar and Hansen 1986). Boemare (1983) also observed that this bacterium species as well as Serratia liquefaciens, P. aureofaciens, P. fluorescens and Aeromonas spp. are pathogenic to G. mellonella.

Pathogenicity of bacteria may not be of fundamental importance for the insect death, since it was already demonstrated that axenic nematodes are pathogenic to insects (Boemare 1983). According to Boemare, the most important role of the bacteria is to provide nutrient for the nematodes. Toxic action of axenic nematodes was also reported by Burman (1982). Akhurst (1986a), on the other hand, observed that Xenorhabdus poinarii and S. glaseri are pathogenic only when they are associated with each other.

Experiment 3, in which doses of X. nematophilus and X. bovienii were inoculated, indicated that a high dose of those bacteria to kill Acheta domesticus. Other experiments to determine the lethal dose of Steinernema scapteriscus to Scapteriscus spp. have shown that high inoculum levels are required to kill 50% of the tested population (Chapter 6). The

reaction of this insect species to S. scapterisci seems to be similar to the reaction of A. domesticus (Nguyen, 1988). It is likely that there is a relationship between the pathogenicty of bacteria reported in this chapter and the infectivity of the nematodes (Chapter 6).

All the infective juveniles of S. scapterisci carried bacteria in monoxenic culture with several bacterial species. More than one bacterium species can be carried by infective juveniles of S. scapterisci as reported in Chapter 2. It is not known whether these associations would induce a different insect reaction or whether the development of one bacterial species would interfere in the development of the other when introduced into the insect hemocoel by the nematodes.

It was also demonstrated in the present work that various nematode/bacterium combinations obtained from monoxenic cultures can be as efficent as nematodes produced in vivo on an insect host. Associations with bacteria isolated from either S. scapterisci or from S. carpocapsae are similarly infectious to Scapteriscus borellii. It is interesting to note that X. nematophilus seems to be quite specific since it has been isolated in nature only from S. carpocapsae.

Perhaps the lack of specificity of bacteria in S. scapterisci gives this nematode a better chance for establishment and survival as has been observed from field releases in Florida (Smart et al. 1990). Even bacteria which are usually not entomopathogens can be so when introduced by

S. scapterisci into the hemocoel. The associations of this nematode species with a variety of bacteria is apparently beneficial.

CHAPTER 5
IN VITRO CULTURE OF STEINERNEMA SCAPTERISCI

Introduction

Soon after Steinernema Travassos 1927, was described (Travassos 1927) and Steinernema (= Neoaplectana) glaseri (Steiner 1929) Wouts, Mracek, Gerdin and Bedding 1982 was found to be a biological control agent with potential to be used against insects, the importance of steinernematid nematodes was visualized, and attempts to culture these nematodes were initiated. Glaser (1931) was the first nematologist to develop a culture method for Steinernema. Working with S. glaseri on a veal infusion agar inoculated with fresh yeast, Glaser obtained nematode reproduction for up to six subcultures. Several other solid media and modifications were subsequently developed (McCoy and Glaser 1936, McCoy and Girth 1938, Glaser 1940). But culture media such as dog food agar (House et al. 1965, Hara et al. 1981) and chicken offal or pig kidney and beef medium (Bedding 1981, 1984) and some chemically defined media (Wouts 1981, Buecher and Popiel 1989) have made culture of species of Steinernema more efficient.

Dutky et al. (1967) determined that S. (=Neoaplectana) carpocapsae requires an exogenous source of sterol for its growth and reproduction. Those authors concluded that although there exists specificity concerning suitability of the different sterols, several of the compounds that they tested, including cholesterol, could fulfill this requirement. They reported that cells of the associated bacteria provided only trace amounts of sterols and it was essential to provide sterol as a medium component. More recently, Buecher and Popiel (1989) were successful in rearing S. feltiae Filipjev strain 42 in a semidefined liquid medium containing 40 μg/ml cholesterol.

Species of Xenorhabdus also are known to present high proteolitic activity (Schmidt et al. 1988), a characteristic which allows for the associated nematodes to grow in several substrata with a high protein content (Bedding 1976). The nematodes not only feed on the bacteria but also absorb nutrients directly from the medium (Devidas et al. 1982).

The definition of a culture medium on which to produce a certain species of nematode depends largely on the amounts of inoculum required, as well as the time, and conditions available (Friedman 1990). According to this author liquid culture represents the best economies in scaled up processes.

Glaser (1940) was apparently the pioneer in the use of liquid medium for rearing steinernematid nematodes. Stoll (1953) followed Glaser's work on S. (Neoaplectana) glaseri in

this type of medium, and developed a method based on the addition of raw liver extract to veal or beef heart infusion. Hansen et al. (1968) reported the growth of S. carpocapsae in a liquid formula. Further improvement in this area was achieved by Buecher and Popiel (1989), who developed a liquid medium for rearing S. feltiae.

Although several authors have succeded in devising new or modified media, the most productive media are those developed by commercial companies. These companies have developed nematode culture techniques mainly in liquid formulas in scaled-up processes, usually involving large capacity fermenters. These procedures are protected by patents and are not available for general use.

As new species or strains of Steinernema are found and or described, the need to determine their efficacy as biological control agents requires the production of inoculum of that particular species or strain. Production might not be possible always by a commercial company. Furthermore, characteristics of a particular nematode species or strain in culture constitutes important information which might contribute to the knowledge of its action in the environment.

The objective of this work was to determine the effect of inoculum density and culture media composition on the development of Steinernema scapterisci in monoxenic culture. Specific experiments examined effect of selected inoculum

densities, effect of cholesterol supplements to the media, and effect of semi-solid media on growth of S. scapterisci.

Materials and Methods

Effect of selected inoculum densities on growth of S. scapterisci on solid medium

The inoculum for this study was obtained from Biosys, Palo Alto, California. This inoculum was cultured on dog food agar (Hara et al. 1981) in 150 x 15 mm test tubes at 27C, and was subcultured on the same medium. The inoculum from several of these test tube cultures were combined, and nematode density was determined by counting five 100 µl samples in a counting dish under a stereoscopic microscope.

Treatments were 10; 50; 100; 250; 500; 1,000; 5,000; and 10,000 nematodes delivered in 0.2 ml deionized water per petri dish. The range of treatment levels was obtained by diluting the original inoculum with sterile deionized water. These initial populations were composed of mixed life stages of adult females, males, and juveniles at different stages of development. The experimental design was a randomized complete block with five replications per treatment and harvesting date. Each replication consisted of one 100 x 12 mm petri dish containing about 25 ml of dog food agar prepared according to Hara et al. (1981). The experiment was conducted under aseptic conditions at all phases with dishes stored in an incubator at 27C.

Separate harvests were performed at 14, 21, and 28 days after inoculation. Nematodes were extracted from the five replications for each harvesting date over a 5-day period according to the procedure described by Hara et al. (1981). Nematodes were collected after one, three and five days and combined to represent the total population obtained for the harvest time at which the extraction was initiated (i.e., 14, 21, or 28 days). Nematode numbers in each replication were determined by counting five 500 μl subsamples from each replication and obtaining the average for each replication. Numbers of infective juveniles, total juveniles, total adults, and total of all stages were computed and transformed by $\log_{10}(x + 1)$ prior to a one-way analysis of variance (Freed et al. 1987). Data for each harvest date were analyzed separately. Log-transformed means of nematode densities at the selected inoculation levels were compared by Duncan's Multiple Range Test at $P = 0.05$, but untransformed means are reported.

Effects of supplementary cholesterol on growth of Steinernema scapterisci on solid medium

The medium used in this work consisted of: 37 g dehydrated brain-heart infusion (Difco Laboratories, Detroit, MI), 15g agar (Fisher Scientific, Fair Lawn, NJ), and 1,000 ml deionized water. This medium was heated, divided into four portions, and three portions supplemented with amounts of cholesterol equivalent to 20, 40, and 60 μg/ml. One portion

was left without cholesterol as a control. The cholesterol was mixed with 1.0 ml of corn oil and heated before being added to the hot medium. The medium then was dispensed in amounts of 8.0 ml into 150 x 15 mm test tubes and autoclaved at 123 C and 20 lbs / sq. in. for 15 minutes.

The inoculum was composed of different stages of S. scapterisci obtained from monoxenic cultures as described in Chapter 2. The bacterial symbiont used was Xenorhabdus nematophilus (Isolate SC01-1) obtained from S. carpocapsae strain All. Nematodes from about 2 week-old cultures in test tubes were collected in sterile deionized water, five subsamples taken to determine the nematode concentration, and aliquots of 20 μl containing an average of 103 nematodes were inoculated into each test tube. Cultures were incubated at 27C and the five replications from each treatment harvested at 5, 10, and 15 days after inoculation. The harvesting procedure consisted of washing the nematodes with two 5.0 ml amounts of deionized water and transferring the nematode suspension to 150 x 24 mm test tubes. Concentration of nematodes was determined from the average of five 10 μl subsamples from each of the five replications. Data for each harvest date were analyzed separately by an analysis of variance, and means separated by Duncan's Multiple Range Test at $P = 0.05$ (Freed et al.). Before harvesting, samples of the bacteria were taken randomly from the cultures and streaked on T7-TTC medium to

confirm the presence of the primary form of X. nematophilus according to the procedure described by Akhurst (1980).

Development of Steinernema scapterisci in three semi-solid culture media

After preliminary observations concerning the suitability of some culture media for the growth of S. scapterisci, three compositions of semi-solid media were chosen for experimentation. The components of these media were as follows: Medium 1: Brain-heart-cholesterol [37 g dehydrated brain-heart infusion (Difco Laboratories, Detroit, MI), 0.02g cholesterol (Eastman Kodak Co., Rochester, NY), 2.0 g agar (Fisher Scientific) 1,000ml deionized water]. Medium 2: Liver extract cholesterol [7.5 g liver extract (Difco Laboratories) dissolved in 100 ml deionized water, 40 g soy peptone (Sigma Chemical Co., St. Louis, MO), 20 g glucose (Sigma Chemical Co.), 10 g yeast extract (Sigma Chemical Co.), 0.02 g cholesterol (Eastman Kodak Co.), 1.0 ml corn oil, 900 ml deionized water]. Medium 3: Dog food extract [100 g Dog food biscuits 100 g (Gaines - Gravy train, beef flavor. Gaines Pet Food Corp., Chicago, IL), 1,000 ml deionized water]. The brain-heart cholesterol medium was prepared following the usual procedures for culture media. The liver extract cholesterol medium was prepared in the same way, except that the liver extract suspension was heated at 50C for 1 hr. and filtered through a Millipore filter (Millipore Filter Corp. Bedford, MI) using a 0.2 μm pore Metricel membrane filter

(Gelman, Sciences, Inc. Ann Harbor, MI). The dog food extract medium was prepared as follows: dog food biscuits were pulverized in a blender, mixed with 500 ml deionized water, blended again for about 1 minute and strained through a kitchen sieve while an additional 500 ml deionized water was added. Each medium was distributed in 500 ml Erlenmeyer flasks and autoclaved at 123C, and 20 lbs / sq. in. pressure for 15 minutes. Upon cooling, aliquots of 50 ml were dispensed in 150-cm^3 tissue-culture flasks.

Nematode inoculum consisted of S. scapterisci grown monoxenically with Xenorhabdus nematophilus (Isolate SC01-1) obtained from S. carpocapsae strain All reared on Galleria mellonella. The inoculum was produced on brain-heart cholesterol agar similar to that described above, but with 15 g instead of 2 g Bacto agar in 150 x 15 mm test tubes, according to the procedures described in Chapter 3. The three different types of culture media in tissue-culture flasks were inoculated initially with 1.0 ml of 24-hour old X. nematophilus cultures grown in nutrient broth (8.0 g Nutrient broth, 1,000 ml deionized water). The cultures were maintained on a platform rocker (Bellco, Vineland, NJ) at room temperature. Twenty four hours after the bacteria were inoculated the three culture media were inoculated, with 1.0 ml of the above nematode suspension containing 1,000 nematodes at different developmental stages. Samples were taken periodically at random with a wire loop and streaked on T-7

TTC, in order to determine the presence of the primary form of Xenorhabdus nematophilus, according to the description reported by Akhurst (1980). Since the rate of nematode development was different in the tested media, as evaluated by direct observation of cultures under a stereoscopic microscope, harvest was done at two different times. Cultures on brain-heart cholesterol agar were processed 20 days after inoculation with nematodes and cultures on liver extract cholesterol and dog food extract were processed 40 days after inoculation with nematodes.

Results

Effect of selected inoculum densities on growth of Steinernema scapterisci on solid medium

The data obtained did not indicate clearly defined differences in population growth among the different initial doses (Tables 5.1, 5.2, and 5.3). Some trends, however, could be observed. Percentage of infective juveniles in relation to total population appeared to be higher at lower doses than at higher doses at the 14-day harvest, 14 days after the inoculation (Table 5.1). Number of adults was lower at lower doses at the first harvest but stabilized at the two subsequent harvests. Total numbers (all stages) were more variable than the separate numbers of infective juveniles and adults because other juvenile stages and pre-adults were

included in the total counts. Nematode production ranged from 209,160 to 1,360,600 total juveniles and from 800 to 113,200 adults.

Effects of supplementary cholesterol on growth of Steinernema scapterisci on solid medium.

Five days after inoculation there were no significant differences in population development resulting from the addition of cholesterol (Table 5.4). The effects of cholesterol became evident 10 days after inoculation, when numbers of infective juveniles at all three doses of cholesterol were significantly different from the control (Table 5.5). Numbers of females were higher than the control at doses of 20 and 40 μg cholesterol; males were not affected. At 15 days after harvest, only at 20 μg/ml cholesterol were the numbers of juveniles, females, total adults, and total nematodes of all stages higher than the control (Table 5.6). Higher doses of this compound were apparently detrimental and the nematode numbers resulting from the addition of 40 and 60 μg cholesterol/ml medium did not differ from the control.

Development of Steinernema scapterisci in semi-solid culture media

The nematode developed on all three media, but infective juveniles appeared earlier in brain-heart-cholesterol medium than in the other media (determined by direct observation - no data shown). Therefore, nematodes in the brain-heart cholesterol medium were harvested after 20 days, and nematodes

in the liver extract cholesterol and dog food extract were harvested 40 days after inoculation.

Nematode production in dog food extract was the highest of the three media tested, and 96.4% of the nematodes were infective juveniles (Table 5.7). Infective juveniles on brain-heart cholesterol represented 81.1% of the total juveniles and 78.6% of the total nematodes all stages. The proportion of infective juveniles on liver extract cholesterol was only 45.6% of the total juveniles, and 43.1% of the total nematodes of all stages. In all experiments, samples taken to verify the presence of phase of the X. nematophilus primary phase produced colonies on T7-TTC medium with the characteristics described by Akhurst (1980) for the primary phase of that species.

Table 5.1 - Population levels of Steinernema scapterisci on dog food agar in petri dishes 14 days after inoculation with eight initial population levels.

Initial population per dish	Numbers of nematodes/petri dish			
	Infective juveniles	Total juveniles	Adults	Total all stages
10	357,000 a	628,300 a	800 a	636,300 a
50	241,860 a	475,800 a	4,890 a	480,690 a
100	154,500 a	509,100 a	42,300 b	551,400 a
250	467,400 a	1,074,200 a	48,200 b	1,122,400 a
500	265,140 a	605,410 a	74,800 b	680,210 a
1,000	301,560 a	787,360 a	64,070 b	851,430 a
5,000	266,400 a	735,600 a	113,200 b	848,800 a
10,000	105,400 a	650,100 a	64,800 b	714,900 a

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple Range Test ($P = 0.05$). Statistical analyses performed on data transformed by $\log_{10}(x + 1)$, but untransformed means are shown.

Table 5.2 - Population levels of Steinernema scapterisci on dog food agar in petri dishes 21 days after inoculation with eight initial population levels.

Initial population per dish	Numbers of nematodes/petri dish			
	Infective juveniles	Total juveniles	Adults	Total all stages
10	252,820 a	705,780 a	58,380 a	786,680 ab
50	291,100 a	902,700 a	16,400 a	938,700 ab
100	69,620 ab	209,160 ab	17,600 a	240,220 bc
250	257,440 a	803,790 a	38,860 a	867,720 abc
500	473,680 a	1,248,960 a	20,880 a	1,296,760 a
1,000	163,200 b	295,750 b	13,440 a	335,070 c
5,000	251,100 ab	738,816 ab	22,048 a	771,340 abc
10,000	200,720 a	470,528 ab	9,180 a	503,020 abc

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple Range Test($P = 0.05$). Statistical analyses were performed on data transformed by $\log_{10}(x + 1)$, but untransformed means are shown.

Table 5.3 - Population levels of Steinernema scapterisci on dog food agar in petri dishes 28 days after the inoculation with eight initial population levels.

Treatments (Initial population)	Average numbers of nematodes/replication			
	Infective juveniles	Total juveniles	Adults	Total all stages
10	460,570 a	822,970 a	25,650 a	874,500 a
50	190,126 a	472,890 a	11,412 a	502,474 a
100	326,440 a	713,440 a	31,740 a	763,800 a
250	374,010 a	969,100 a	25,040 a	1,015,300 a
500	232,712 a	540,548 a	25,336 a	580,288 a
1,000	270,216 a	639,516 a	54,180 a	684,164 a
5,000	793,590 a	1,360,600 a	23,840 a	1,398,390 a
10,000	470,850 a	747,670 a	11,560 a	770,030 a

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple Range test ($P = 0.05$). Statistical analyses were performed with data transformed by $\log_{10}(x + 1)$, but untransformed means are shown.

Table 5.4 - Effects of supplementing brain-heart agar with cholesterol on the numbers of *Steinernema scapterisci* 5 days after inoculation.

Levels of cholesterol (µg/ml)	Numbers of nematodes/replication				
	Juveniles	Females	Males	Total adults	Total all stages
0	3,024 a	76 a	84 a	160 a	3,184 a
20	4,216 a	152 a	96 a	248 a	4,456 a
40	3,656 a	132 a	184 a	316 a	3,972 a
60	3,156 a	116 a	88 a	204 a	3,360 a

Data are means of five replications. Means in columns followed by the same letter are not different according Duncan's Multiple Range Test ($P = 0.05$).

Table 5.5 - Effects of supplementing brain-heart agar with cholesterol on the numbers of *Steinernema scapterisci* 10 days after inoculation.

Levels of cholesterol (µg/ml)	Numbers of nematodes/ replication*				
	Juveniles	Females	Males	Total adults	Total all stages
0	2,616 b	280 b	216 a	496 b	3,200 b
20	8,100 a	740 a	412 a	1,152 a	9,252 a
40	8,240 a	584 a	276 a	860 ab	9,372 a
60	6,928 a	484 ab	412 a	896 a	7,824 a

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple Range Test ($P = 0.05$).

Table 5.6 - Effects of supplementing cholesterol to brain-heart agar on the numbers of Steinernema scapterisci 15 days after inoculation.

Levels of cholesterol µg/ml	Numbers of nematodes/replication				
	Juveniles	Females	Males	Total adults	Total all stages
0	5,252 b	584 b	568 a	1,152 b	6,404 b
20	18,820 a	2,060 a	1,024 a	3,084 a	21,864 a
40	11,200 ab	1,068 b	724 a	1,792 b	12,992 ab
60	8,024 b	800 b	592 a	1,392 b	9,416 b

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple Range Test ($P = 0.05$).

Table 5.7 - Numbers of *Steinernema scapterisci* per flask developing on three different culture media.

Culture medium	Infective juveniles	Total juveniles	Total adults	Total all stages
Brain heart cholesterol*	411,025 b	506,737 b	16,360 b	523,096 b
Liver extract cholesterol**	302,190 b	662,604 b	38,747 a	701,351 b
Dog food extract**	1,863,537 a	1,926,227 a	6,788 b	1,933,015 a

Data are means of five replications. Means in columns followed by the same letter are not different according to Duncan's Multiple range Test ($P = 0.05$).

* - Nematodes harvested 20 days after inoculation.

** - Nematodes harvested 40 days after inoculation.

Discussion

The nematode population obtained on dog food agar in petri dishes was composed mostly of juveniles at different developmental stages. In the first harvest, 14 days after inoculation, the proportion of infective juveniles in relation to the total number of juveniles tended to be higher at the lower initial population levels. High numbers of adults migrated through the circles of filter paper and into the formalin solution in the collecting dishes and were collected together with the juveniles. Other Steinernema species seem to behave differently under similar conditions. Hara et al.(1981) obtained only infective juveniles of Steinernema (=Neoaplectana) carpocapsae All strain in the formalin in the collecting petri dishes, indicating that only nematodes at this stage migrated to the solution. Migration of adults of Steinernema scapterisci out of the cadaver has been observed constantly in in vivo cultures. Differently than reported for other steinernematid nematodes reared on a host and collected according to the method of White (1927) and Poinar (1979), adults of S.scapterisci migrate across the filter paper and into the water in collecting dishes. Adults exiting the culture medium probably influenced the sequence of nematode production in the population dynamics experiment. Migration occurred in all treatments and many adults were collected together with juveniles, mainly in high doses. The proportion of adults in the collecting dishes was lower, however, in

these treatments than in the lower doses. Various life stages of the nematodes also were observed on the petri dish lids during the period of incubation of the cultures. The migration of various life stages, not just infective juveniles may have caused the high variability observed in this experiment and also for the lower final populations of infective juveniles when compared with numbers of infective juveniles obtained by Hara et al. (1981). In general, there were no consistent significant differences ($P = 0.05$) in numbers of infective juveniles among the eight inoculun levels tested at the three harvest dates. Similar results were obtained with S. carpocapsae on pig kidney medium by Popiel et al. (1986) who reported that production of infective juveniles was independent of the inoculum level.

Since inoculum density did not affect ($P = 0.05$) numbers of nematodes produced and since numbers of infective juveniles 14 days after inoculation tended to be higher with lower initial populations, it is here suggested that inocula ranging from 10 to 250 nematodes per dish could be used for most efficient nematode production in an incubation period of two weeks. Quality of S. scapterisci produced on dog food agar in petri dishes, however, seems to be inferior because adults collected in the formalin suspension together with juveniles, do not survive well in storage and the resulting decomposition of adults decreases survival of juveniles. An alternative would be to collect the infective juveniles using a modified

Baermann funnel procedure (Nguyen 1988) but this would add one more operation to the extraction procedure.

Significant increases in production of S. scapterisci in response to the addition of cholesterol were observed 10 and 15 days after inoculation. At 10 days, all treatments containing cholesterol were significantly different from the control (no cholesterol), but at 15 days, only 20 μg/ml cholesterol induced a significant increase in nematode production in relation to the control. Addition of cholesterol at that concentration (20 μg/ml) is beneficial to the growth of S. scapterisci on brain-heart medium, whereas excessive levels of cholesterol cannot be recommended.

Since steinernematids absorb nutrients directly from the medium, probably because of the action of associated bacteria on the medium components, additives may enhance nematode production in vitro (Friedman 1990). As demonstrated by Dutky et al. (1967), S. carpocapsae also requires cholesterol for its reproduction since the associated bacteria provide only minor amounts of that chemical. Sterol concentrations as low as 0.1 to 2.0 μg/ml may be enough for some nematodes (Hieb and Rothstein 1968, Lu et al.1977). Although Buecher and Popiel (1989) used only one concentration of cholesterol (40 μg/ml), they obtained good reproduction of S. feltiae. In our studies, it was shown that S. scapterisci grows best over 15 days on brain-heart medium when 20 μg/ml cholesterol is added to the medium. This effect was not readily shown as indicated by the

harvest at five days after inoculation probably because X. nematophilus as well as the medium components provide this chemical. Brain-heart agar is an oligidic medium whose composition is only partially defined. Cholesterol is probably present in this product but the amounts seem to be inadequate for reproduction of S. scapterisci. Although the amounts obtained from the medium as well as from the bacteria may induce initial nematode growth, it is not sufficent to maintain nematode development for a more prolonged period of time. The improved culture medium as reported here is suitable for the production of basic inoculum to be used for production of larger numbers of nematodes and also for use in laboratory studies.

Although S. scapterisci was able to grow in all the three culture media tested, development was best on dog food extract. Development on this medium was delayed when compared to brain-heart cholesterol medium, but the number of infective juveniles was 4.5 times higher than in the latter medium. Altough the composition of brain-heart-infusion is not determined, this medium is probably poorer than dog food hence the earlier and more limited formation of infective juveniles. On dog food medium, the richer composition allowed for continuous life cycle instead of formation of the infective stage which does not feed. Reproduction of S. scapterisci on liver-extract cholesterol medium was similar to the multiplication in brain-heart cholesterol medium, but required

a longer period of incubation. In addition, the former is a more expensive medium and the preparation is more complex. Brain-heart cholesterol medium is a relatively simple culture medium that can be used for nematode production in an incubation period of 20 days. Dog food extract medium can be used when the period of incubation can be extended.

CHAPTER 6
PATHOGENICITY OF STEINERNEMA SCAPTERISCI TO SELECTED INSECTS IN THE ORDER ORTHOPTERA

Introduction

Steinernema scapterisci was isolated from infested mole crickets (Scapteriscus vicinus) under natural conditions in Uruguay and introduced into Florida in 1985 (Nguyen and Smart 1988, 1990a). It has proved to be an efficient biological control agent of mole crickets in the genus Scapteriscus in Florida and has become established in several release sites (Frank et al. 1988, Parkman and Frank 1992, Smart et al. 1990). The ability of S. scapterisci to carry several bacteria of various genera and species as symbionts may be an important factor in its establishment in the field (Chapters 2, 3, and 4).

Nguyen (1988) conducted laboratory experiments to determine the susceptibility of the three species of Scapteriscus occurring in Florida to S. scapterisci. Only one inoculum level was used in those experiments with multiple hosts per arena. Other insects in the Order Orthoptera, and in a few other orders were tested under the same conditions.

The objectives of this work were to determine experimental conditions under which to test for pathogenicity

of S. scapterisci to selected insects in the Order Orthopterea and to determine the inoculum levels required to kill those insects.

Material and methods

Production of inoculum of Steinernema scapterisci

Infective juvenile nematodes for all the experiments were obtained by rearing the nenatode alternately on mole crickets (Scapteriscus borellii and S. vicinus) and house crickets (Acheta domesticus). The species of Scapteriscus were obtained from field collections in various sites in Florida. House crickets were obtained from a local fish bait store. Nematode production was achieved by using the procedure of Dutky et al. (1964) as reported by Poinar (1979) for the production of S. carpocapsae DD-136 on Galleria mellonella. Nematode inoculum was stored in Erlenmeyer flasks at 8C and used within a period of two months.

Pathogenicity to Scapteriscus spp.

Preliminary tests were performed to establish a system to test pathogenicity to mole crickets. Small plastic petri dishes (35 x 10 mm) lined with one-42.5 mm Whatman No. 2 filter paper (Whatman International Ltd., Maidstone, England) were inoculated with 1.0 ml inoculum in dosages ranging from 1 to 32 infective juveniles. Previously anesthetized insects were transferred to the nematode-inoculated petri dishes, lids were applied, taped and dishes incubated at 25C. This test was

discontinued because the mole crickets rapidly chewed the filter paper and insect mortality was low and inconsistent.

Based on these observations, another system and another range of inoculum levels was exsmined. The arena consisted of plastic vials 5.4-cm-long x 2.7-cm with snap caps, containing 9.0 g substrate (98% sand, 0% silt, 2% clay). This substrate was sterilized at 123C at 20 lbs / sq. in. for 30 minutes, and dried in an oven at 100C until constant weight was obtained. Inoculum consisted of infective juveniles obtained from in vivo cultures. Two experiments were conducted in this system. Experiment 1: The treatments consisted of 0, 100, 500, 1,000, and 10,000 infective juveniles delivered in 1.0 ml deionized water per container. Scapteriscus vicinus was the insect species tested.

Experiment 2: The treatments consisted of 0, 100, 1,000, 2,000, 4,000, 8,000, and 10,000 infective juveniles delivered in 1.0 ml deionized water. Scapteriscus borellii was the insect species tested.

In both experiments, one insect was placed into each container immediately after the nematodes were added. Insects were anesthetized previously by exposure to CO_2 for a few minutes. The dishes were maintained at 25C. The experimental design was a randomized complete block with six replications of five insects each. Host mortality was assessed at 24, 48, i72, and 96 hours after inoculation. Mortality data were

submitted to analysis of variance, and means were separated by Duncan's Multiple Range Test (Freed et al. 1987).

Pathogenicity to Gryllus rubens

The type of arena, substratum, and inoculum were similar to the experiments with Scapteriscus spp. The treatments were 0, 10, 100, 1,000, 5,000, and 10,000 nematodes per replication delivered in 1.0 ml deionized water. The experimental design was a randomized complete block with five replications of five insects each. Incubation conditions, mortality assessment, and data anlyysis were as in the previous experiments.

Pathogenicity to Romalea guttata

Two experiments were conducted with this insect. The arenas consisted of 5.4 cm x 2.7 cm plastic vials with filter paper lining the inner surface except for a longitudinal hiatus of approximately 0.5 cm. After the nematodes and one insect were introduced into each vial, the top was covered with two layers of cheesecloth held in place with a rubber band. Vials were maintained at 25C inside plastic bags which were open at the top.

Experiment 1: Treatments were 0, 100, 1,000, and 10,000 nematodes per replication, delivered in 0.5 ml of deionized water. The experimental design was a randomized complete block with 6 replications of 5 insects each. Mortality was determined at 24, 48, 72, and 96 hours.

Experiment 2: Methods were identical to those for the first experiment with this insect species, except that treatments were 0, 100, and 1,000 nematodes per vial.

Results

Pathogenicity to Scapteriscus spp.

Experiment 1: Mortality in treatments with inoculum levels of $\leq$ 1,000 nematodes was low and did not differ significantly from the 0 inoculum level at any exposure period times (Table 5.1). Mortality in the 10,000 nematode treatment was significantly different from all other treatments at all exposure periods. Mortality reached the 50% level after 48 hours and 80% after 96 hours.

Experiment 2: Percent mortality 24 hours after inoculation was higher ($P = 0.05$) in the 8,000 and 10,000 nematode treatments than for all other treatments (Table 6.2). At 72 and 96 hours, percent mortality was significantly different from the 0 inoculum control at levels of $\geq$ 2,000 nematodes. The 50% mortality level was reached within 48 hours with an inoculum level of 10,000 nematodes and within 72 hours with initial doses of 4,000 or 8,000 nematodes.

Pathogenicity to Gryllus rubens

Significant differences in mortality from the 0 dose control were obtained with doses of $\geq$ 1,000 nematodes at 24 hours (Table 6.3). Only 10,000 nematodes induced mortality of 50%, taking into account the mortality in the control. At 48

and 72 hours all the nematode doses gave mortality significantly higher than the control but mortality in the control was very high. The high mortality in the controls indicate that the conditions of the test were not satisfactory.

Pathogenicity to Romalea guttata

Experiment 1. Mortality greater than that of the control was obtained with 10,000 nematodes at 24 hours (Table 6.4). Mortality above 50% was obtained with treatments of 1,000 and 10,000 nematodes at 48, 72, and 96 hours after exposure (Table 6.4).

Experiment 2. With inoculum levels of 100 and 1,000 nematodes, mortality levels in this experiment were generally similar to those observed in Experiment 1 (Table 6.5). However, mortality levels of 50% or greater were achieved 24 hours earlier in Experiment 1 than in Experiment 2 for the 100 and 1,000 nematode inoculum levels.

Table 6.1. Percent mortality of Scapteriscus vicinus from Scapteriscus scapterisci inoculum in laboratory tests. Experiment 1.*

Inoculum level	Time after exposure (hours)			
	24	48	72	96
0	6.7 a	6.7 a	10.0 a	13.3 a
100	3.3 a	10.0 a	16.7 a	20.0 a
500	6.7 a	16.7 a	26.7 a	33.3 a
1,000	3.3 a	16.7 a	30.0 a	30.0 a
10,000	33.3 b	56.7 b	76.7 b	80.0 b

* - Data are percent mortality from five replications composed of five insects each. Means in columns followed by the same letter are not significantly different ($P = 0.05$).

Table 6.2. Percent mortality of *Scapteriscus borellii* from *Steinernema scapterisci* inoculum in laboratory tests. Experiment 2.*

Inoculum level	Time after exposure (hours)			
	24	48	72	96
0	0.0 a	3.3 a	6.7 a	6.7 a
100	0.0 a	0.0 a	6.7 a	6.7 a
1,000	3.3 a	6.7 a	16.7 ab	20.0 ab
2,000	0.0 a	10.0 a	36.7 cb	40.0 bc
4,000	6.7 a	33.3 b	50.0 cd	56.7 cd
8,000	33.3 b	43.3 b	66.7 de	66.7 de
10,000	30.0 b	63.3 c	73.3 e	80.0 e

* - Data are percent mortality from 5 replications consisting of 5 insects each. Means in columns followed by the same letter are not significantly different ($P = 0.05$).

Table 6.3. Percent mortality of Gryllus rubens from Steinernema scapterisci in laboratory tests.*

Inoculum level	Time after the inoculation (hours)			
	24	48	72	96
0	9.0 a	22.0 a	32.0 a	64.0 a
10	20.0 ab	56.0 b	48.0 b	76.0 ab
100	24.0 abc	58.0 b	76.0 c	88.0 bc
1,000	40.0 bcd	75.0 bc	84.0 c	96.0 c
5,000	52.0 cd	96.0 c	100.0 d	96.0 c
10,000	64.0 d	92.0 c	100.0 d	100.0 c

* - Data are percent mortality from 5 replications composed of 5 insects each. Means in collumns followed by the same letter are not significantly different ($P = 0.05$).

Table 6.4 - Percent mortality of Romalea guttata from Steinernema scapterisci in laboratory tests. Experiment 1.*

Inoculation level	Time after exposure (hours)			
	24	48	72	96
0	0.0 a	0.0 a	10.0 a	16.7 a
100	0.0 a	33.3 b	50.0 b	53.3 b
1,000	0.0 a	66.7 c	80.0 c	80.0 c
10,000	33.3 b	100.0 d	100.0 c	100.0 c

* - Data are percent mortality from 6 replications composed of 5 insects each. Means followed by the same letters are not significantly different (P =0.05).

Table 6.5. Percent mortlality of Romalea guttata from Steinernema scapterisci inoculum in laboratory tests. Experiment 2.*

Inoculum level	Time after exposure (hours)			
	24	48	72	96
0	0.0 a	10.0 a	15.0 a	15.0 a
100	0.0 a	35.0 a	40.0 ab	50.0 b
1,000	0.0 a	30.0 a	65.0 b	70.0 b

* - Data are percent mortality from 4 replications composed of 5 insects each. Means in columns followed by the same letter are not significantly different ($P = 0.05$).

Discussion

The inoculum levels in which significant mortality of *Scapteriscus* spp. occurred are considered high. From the results in Experiment 1, it is concluded that a nematode concentration above 1,000 per insect is necessary to cause significant mortality to *S. vicinus* , in this case with a dose of 10,000 nematodes per insect. However no dosages between 1,000 and 10,000 nematodes were tested. Mortality at 48-, 72-, and 96 hours did not change significantly from that at 24 hours indicating that mortality readings taken at 24 hours were sufficient to indicate pathogenicity. In Experiment 2, significant mortality of *s. borellii* was obtained with doses of 4,000 nematodes and higher, starting at 48 hours after exposure. Increased mortality was observed in readings taken after 48 hours. Doses of 2,000 nematodes and higher, were enough to induce significant mortality to this host at 72 hours. The results of this experiment were consistent with those obtained in Experiment 1. With more doses within a certain range of concentrations, observations should be extended for a longer period of time, such as 96 hours, to obtain a more precise reaction.

It is difficult to compare results from the literature with those obtained in these experiments, which were conducted in sand rather than on filter paper because of the chewing habit of mole crickets. As observed in preliminary tests, soon after exposure, the insects may not be in contact with the

nematodes, most of which are in the crumpled filter paper balls. Mole crickets are typically active soil dwellers. Infective juveniles of Steinernema spp. are also known to be active in the soil although active dispersal by the nematodes themselves is low, and influenced by soil texture, moisture, temperature, and the level of activity characteristic of the nematode species (Ishibashi and Kondo 1990). Active migration of S. scapterisci has been observed on water agar cultures covered with a glass coverslip (Esser 1992) and in flasks with culture medium (Chapter 5). The nematode is also likely to be active in the soil environment. Experiments conducted in soil are preferred over filter paper to test the pathogenicity of this nematode to Scapteriscus. Both the nematodes and this host insects are in contact with the substratum in which they live. Also, tests conducted on filter paper are considered too far from the reality (Bedding 1990).

With G. rubens, similarly to the results obtained with Scapteriscus spp., the dosage required to kill this insect was high when compared to the dosage of other species of Steinernema required to kill other hosts. Although significant mortality was obtained with a dose of 1,000 nematodes at 24 hours and with a dose of 10 nematodes at 48 hours, mortality of 50% was obtained only with 5,000 and 1,000 nematodes at 24 and 48 hours, respectively. The conditions in this experiment were unfavorable to G. rubens as indicated by the high mortality of control insects.

Romalea guttata seems to be more susceptible to S. scapterisci than Scapteriscus spp. and G. rubens, since lower doses were required to kill 50% of the population of R. guttata than the latter insect species. However, the arenas were different and this might have been the cause of the differences observed. Conditions in the experiment seemed to be adequate for this insect species.

Previous experiments with S. scapterisci and Scapteriscus involved either high dosages or multiple insects per arena or both (Nguyen 1988, Hudson and Nguyen 1989). The results of the experiments reported here indicate that the dosages necessary to kill 50% of the population in laboratory tests is high for the insects tested. Gaugler et al. (1990) indicated that, in general, there is a low probability of infective juveniles successfully penetrating and establishing even in a highly susceptible host. Interestingly, S. scapterisci has become established in Florida rather quickly after its introduction from Uruguay (Smart et al. 1990). It is not known how defense mechanisms of the insect influenced the nematode action. The association with several species of bacteria reported in Chapters 2, 3, and 4 is likely to play an important role in the relationships of this nematode with the hosts, although no differences in pathogenicity were observed when a high concentration of infective juveniles obtained in monoxenic cultures with some of the bacterium species was inoculated in Acheta domesticus. It seems that the ability of S. scapterisci

to grow and reproduce at the expense of various bacteria compensates for the high numbers required to kill the hosts. If this is true, it might explain the great adaptability of this nematode in the new environment despite the high doses required to infect a host.

CHAPTER 7
GENERAL DISCUSSION

The objectives of this dissertation were related to the biology and culture of Steinernema scapterisci, an entomophagous nematode introduced into Florida in 1985 to help control mole crickets in the genus Scapteriscus (Smart et al. 1990). Studies were conducted to determine what bacteria are associated with the nematode, whether the nematode could be cultured on the bacteria isolated from the nematode, and on the pathogenicity of the nematode/bacterial associations to selected insects in the Order Orthoptera. Pathogenicity of the bacteria alone was determined by injecting them into the hemocoel of mole crickets and house crickets. The nematode was cultured on artifical media in order to produce sufficient quantities for field studies. The pathogenicity of various levels of inoculum of S. scapterisci to selected insects was also determined.

Very surprising results were obtained concerning bacterial associations with S. scapterisci. From the literature it is known that infective juveniles in the genus Steinernema carry specific bacterial symbionts in the genus Xenorhabdus inside a vesicle located at the anterior end of the intestine (Poinar 1979). There is some specificity in

these associations since not all nematode species reproduce with symbionts of other species in the same genus, and usually a given Steinernema species retains only its own symbiont (Akhurst 1983b).

Bacteria were isolated from infective juveniles of S. scapterisci from various sources, and the bacteria were identified by gas chromatography of fatty acids and by biochemical and Gram staining tests. These identifications demonstrated that S. scapterisci can carry several species of bacteria. In addition to the expected Xenorhabdus spp., including X. bovienii, we isolated Ochrobactrum anthropi, Pseudomonas aureofaciens, Xanthomonas maltophilia, and an unknown species related to Paracoccus denitrificans. When these bacterial species were inoculated onto culture media and axenic first-stage juveniles of S. scapterisci added, the nematodes grew and reproduced in monoxenic associations with each of the bacterial species. Three other bacteria, Xenorhabdus nematophilus, symbiont of S. carpocapsae , P. fluorescens Biovar B, and Escherichia coli, not obtained from S. scapterisci but related to those isolated from it were also tested in monoxenic cultures; the nematode grew and reproduced on each of those bacteria.

Reproduction of the nematode was especially good on Pseudomonas aureofaciens, but not statistically different than that on Xenorhabdus nematophilus and P fluorescens. It is interesting to note that X. nematophilus has been reported

only in association with S. carpocapsae under natural conditions. Although there were differences among them, all the bacterial species tested were retained by infective juveniles of the nematode. The number of bacterial cells retained by each infective juvenile ranged from 5 to 60. Other species of Steinernema have been found associated with bacteria other than Xenorhabdus (Poinar 1966, Lysenko and Weiser 1974, Boemare 1983, Boemare et al. 1983). However, those associations were regarded as occasional and irrelevant in the biology of the nematode (Akhurst 1983b). In fact, work done with S. carpocapsae showed that bacteria other than Xenorhabdus can be used as a source of food by the nematode but cells are not retained by juveniles (Akhurst 1983b). Although pathogenicity of the nematode may not be affected, since even axenic nematodes are pathogenic (Boemare 1983), survival of the nematodes is likely to be affected in associations in which bacteria are not retained. Therefore, the finding that S. scaperisci not only feeds but also reproduces on several species of bacteria is significant. This may explain the rapid establishment of S. scapterisci in Florida (Smart et al.).

Pathogenicity tests with the bacteria isolated from S. scapterisci and from other sources showed that they are pathogenic to the mole cricket, Scapteriscus borellii and to the house cricket, Acheta domesticus. Bacterial species not obtained from the nematodes may be as pathogenic to insects as

those species found in association with the nematode. It is known from the literature that non-pathogenic or weakly pathogenic bacteria may cause insect death when introduced into the hemocoel (Boemare 1983).

Preliminary observations had shown that S. scapterisci reproduces poorly on bacteria grown on brain-heart agar medium, a medium usually suitable for production of other species of Steinernema. An experiment was performed to determine the effects of adding cholesterol to brain-hear agar medium. Doses of 20, 40, and 60 µg cholesterol per milliliter of medium were compared with medium without cholesterol. Final populations of S. scapterisci were highest in the treatment with 20 µg cholesterol, indicating that this level of cholesterol is optimal for nematode reproduction.

A population dynamics study was conducted on dog food agar. Initial population levels used in this experiment ranged from 10 to 10,000 nematodes per petri dish. It was demonstrated that low initial populations can produce final populations similar to those obtained with high initial populations. Adult migration during the process of harvesting from this solid medium is likely to have influenced the resuts, which showed considerable variability within treatments.

Three culture medium compositions were tested for reproduction of S. scapterisci. These culture media were all in a semi-solid stage, from which it is easier to extract the

nematodes than from solid media. Infective juveniles were produced earlier on brain-heart-cholesterol medium than on the other media, and harvest could be done after an incubation period of only 20 days. Production of infective juveniles was delayed in dog food extract and in liver- cholesterol extract when compared to the previous medium. Harvest from these two media was done after 40 days of incubation when infective juveniles represented most of the population. Nematode production was highest in dog food extract.

The degree of pathogenicity of S. scapteriscus to some insects in the Order Orthoptera was also determined. Mortalitity of Scapteriscus borellii and S. vicinus was achieved with doses of 4,000 or more infective juveniles per insect in an arena consisting of vials containing sterilized sandy soil. Mortality of the field cricket, Gryllus rubens, was obtained with a dose of 1,000 infective juveniles per insect in the same type of arena above. Mortality of the grasshopper, Romalea guttata, was also achieved with 1,000 infective juveniles per insect. In the latter case, arenas consisted of vials lined with filter paper.

In general, the results of these studies indicate that Steinernema scapterisci behaves differently than other species of Steinernema. Steinernema scapterisci does not require a specific bacterial symbiont, but can use several species of bacteria as symbionts.

Doses of infective juveniles necessary to kill selected insects in the Order Orthoptera are high when compared to other Steinernema species on other susceptible hosts. However, the lack of specificity concerning bacterial symbionts seems to play an important role in nematode infectivity and survival in the field, as has been observed from field releases not related to this research.

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BIOGRAPHICAL SKETCH

Marineide M. Aguillera obtained her Bachelor of Science Degree in Agricultural Sciences at the Universidade Federal Rural do Rio de Janeiro, Brasil, in 1968. Her interest in Nematology led her to the College of Agriculture, University of Sao Paulo, Piracicaba, Sao Paulo, Brasil. There she spent one year specializing in taxonomy of plant-parasitic nematodes under the guidance of Dr. Luiz G. E. Lordello, the eminent nematologist designated as "Father of Brazilian Nematology". She received her Master of Science Degree in Plant Pathology in 1976 at the same University, still under the direction of Dr. Lordello.

Mrs. Aguillera worked as a Plant Pathologist/Nematologist for the Instituto do Acucar e do Alcool, Programa Nacional de Melhoramento da Cana de Acucar from 1972 until 1991, when she obtained a faculty position at the Universidade Federal de Sao Carlos, Araras, SP. In 1988, Mrs. Aguillera enrolled at the University of Florida as a graduate student in the Entomology and Nematology Department and is presently a candidate for the Doctor of Philosophy degree in nematology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Grover C. Smart Jr., Chair
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert McSorley
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joseph H. Frank
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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